

AN INVESTIGATION INTO THE MECHANISM OF  
ACTION OF NITROPRUSSIDE ON ISOLATED  
CARDIOVASCULAR TISSUES

Gordon D. Kennovin

A Thesis Submitted for the Degree of PhD  
at the  
University of St Andrews



1989

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by

Gordon D. Kennovin





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## ABSTRACT

The effect of photolysis of nitroprusside was investigated in both frog ventricular trabeculae and rabbit ear arterial strips. Unphotolysed nitroprusside failed to elicit any effect on frog ventricular twitch tension. However, upon photolysis it had a potent negative inotropic action. The extent of twitch depression was shown to depend on the degree of photolysis. It was postulated that these effects are due to a labile physiologically active photolytic product. This was positively identified as nitric oxide. Preliminary results of the negative inotropic action of thiols and synthesised nitrosothiols are also presented. In contrast to frog ventricle, intact nitroprusside does exert a relaxing effect on precontracted mammalian smooth muscle. This effect is markedly potentiated by photolysis. It is concluded that the mechanism of action of nitroprusside on both tissues involves the release of nitric oxide which is postulated to activate guanylate cyclase. This suggests that mammalian vascular smooth muscle has a mechanism for degrading nitroprusside which is absent in frog ventricle.

I Gordon David Kennovin hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

signed

Date ...14/11/88...

I was admitted to the Faculty of Science of the University of St.Andrews under Ordinance General No. 12 on 16th October 1981 and as a candidate for the degree of Ph.D. on 16th October 1981.

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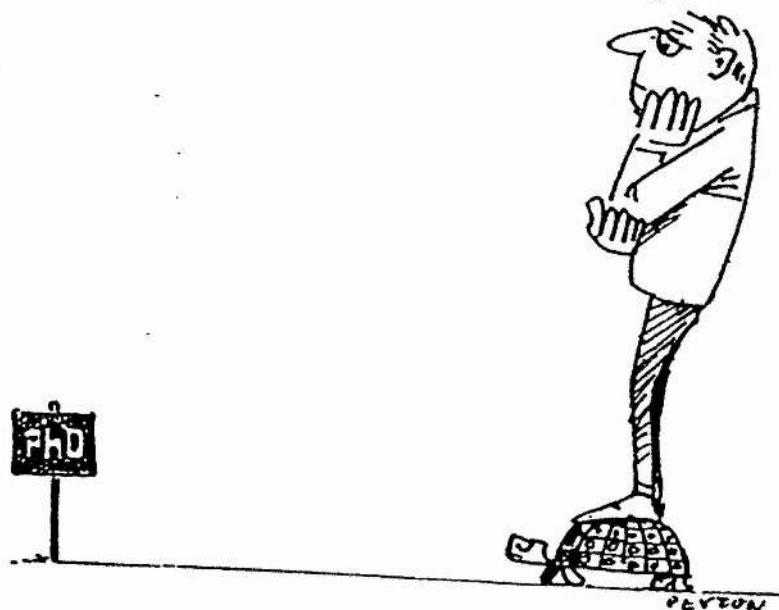
Listening to both sides of a story  
will convince you that there is  
more to a story than both sides.

Frank Tyger

"The first million years were the worst."

Marvin, the paranoid android.  
c/o. Hitch-Hikers guide to the Galaxy.

Douglas Adams



### Acknowledgements

I would like to express my sincere thanks to Dr. Eric Flitney for the help, advice and friendship he has given to me over the years.

I am also indebted to my colleagues 'Jim' Honeyman and Dr 'Jules' Eastwood, for their excellent technical help, encouragement, friendship and witticisms. The scientific and technical staff of the physiology department must also receive my heartfelt thanks.

A special thanks must go to Jim, James, John and the rest of the staff at Kate's. 'Cheers lads'.

Finally, my warmest thanks must go to Aileen for the many cuddles and curries.

Thankyou one and all.



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CHAPTER 1.

Literature review

## INTRODUCTION

Despite almost a century and a half of scientific investigation, nitroprusside (NP) is still a subject of much controversy today. Questions about its structure, electron distribution, chemical properties, photolysis and reaction sequences still remain. Its clinical use in the management of hypertension is still under dispute due to its potential toxicity. The cellular mechanism which underlies its antihypertensive effect has yet to be fully elucidated. Over the years it has been used as an agent in chemical and pharmaceutical analysis, a therapeutic agent for hypertension and cardiac failure, and more recently as a pharmacological tool to specifically raise the intracellular concentration of cyclic guanosine monophosphate (cGMP) in a wide variety of tissues. In this last context, NP was first employed in this laboratory to test a hypothesis concerning the role of cGMP in frog cardiac muscle. Aware of the photosensitivity of the drug, these experiments were conducted in complete darkness. Unexpectedly, a response to NP could only be elicited if the room lighting was switched back on. This result led to a more detailed investigation of the mechanism of action of NP on both frog cardiac and mammalian smooth muscles, the results of which are presented in this thesis.

As there have been several specific reviews on various facets of NP (Rucki, 1977; Van Loenen & Hofs Kemper, 1978; Kreye, 1980; Leeuwenkamp, van Bennekom, van der Mark & Bult, 1984; Butler & Glidewell, 1987), this introduction will concentrate on those aspects which are relevant to its physiological action.

## History

Nitroprusside was first prepared by the St. Andrews chemist Playfair in 1849. He noted that it reacted with a wide variety of chemicals to produce vividly coloured solutions, and stated that it was "by far the best test for the presence of a sulphuret". Similar investigations led to its early use in pharmaceutical analysis for the detection of sulphite (Boedecker, 1861); ketone bodies in the urine of diabetics (Legal, 1883); and primary and secondary aliphatic amines (Simon, 1897; Rimini, 1898) (see refs. in Leeuwenkamp et al, 1984 & Swinehart, 1967). By 1961, numerous colour reactions with a wide variety of organic and inorganic substances had been reported (Swinehart, 1967). The characteristic colour produced by the addition of NP to thiol (-SH) containing compounds led to its widespread use in the determination of glutathione and cysteine in blood (Vesey & Batistoni, 1977). Ketone bodies in serum and urine are still being measured using NP (Vesey & Batistoni, 1977).

The hypotensive action of NP was described as early as 1887 by Davidsohn in his PhD thesis. It is perhaps indicative of how little read PhD theses are that it was not until some 40 years later that Johnson performed the first clinical trial. He reported that NP lowered the blood pressure of a hypertensive patient with no undesirable side effects. Tests on a variety of laboratory species showed that NP was 50-1000 times more potent in reducing blood pressure than nitrite, although the vascular effects of the two anions were otherwise similar (Johnson, 1929).



Modern medical interest was re-kindled by Page and his colleagues (1955) who described the cardiovascular effects of NP on both animals and hypertensive patients. In this study, NP was given orally to hypertensive patients for periods of up to two years. It was also the first study to administer NP intravenously, and showed that its hypotensive action was much greater than with oral administration (Page, Corcoran, Dustan, & Koppanyi, 1955). Symptoms of hypertensive encephalopathy were completely alleviated by constant infusion of NP for up to 14 days, with no deleterious effects. The ability to reduce arterial pressure to a constant level by alteration of the infusion rate, led to NP being introduced in the 1960's as an agent to induce hypotension during surgical procedures under anaesthesia (Jones & Cole, 1968). In 1976, the introduction of sodium nitroprusside under the brand names Nipride (Roche) or Nipruss (Pharma-Schwarz) established its widespread use in the treatment of hypertension. Recently however, its use has been severely restricted by reports that it releases cyanide upon intravenous infusion (Smith & Krusyna, 1974; Vesey & Batistoni, 1977; Smith, Aitken, West, Peterson & Posnanski, 1977; Arnold, Longnecker & Epstein, 1984). Several deaths have occurred through cyanide poisoning following NP administration (Merrifield & Blundell, 1974). To counter this, low infusion rates and/or concomitant infusion of cyanide antidotes have been recommended (Kreye, 1980). Despite this, its therapeutic advantages have led to NP being called the 'drug of choice' for inducing hypotension during surgery (Kreye, 1980).

### Chemistry

### i. Structure

Sodium nitroprusside, sodium nitroprussate and sodium nitroferricyanide are all common names for the inorganic salt disodium nitrosylpentacyanoferrate(2-)-dihydrate. This has the formula  $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO} \cdot 2\text{H}_2\text{O}$ , a molecular weight of 297.9, and is normally formed as dark ruby red crystals. The structure of the nitroprusside anion as determined by X-ray diffraction, infrared and Raman analysis is shown in figure 1:1 (Rucki, 1977; Butler & Glidewell, 1987; Leeuwenkamp, Van Bennekom, Van der Mark & Bult, 1984). Although there is some controversy over the exact molecular orbital distribution of the electrons (Rucki, 1977; Leeuwenkamp et al, 1984), it is generally agreed that the unpaired electron of the NO group shifts towards the central metal ion thus resulting in a nitrosyl group with a formal charge of +1 and a ferrous ion (+2). The nitrosyl group is susceptible to attack from nucleophilic agents (ie bases), which explains the high reactivity of NP with a wide variety of these agents. It is this bond which is also susceptible to photolytic cleavage.

### ii. Photochemistry

The unpaired electron of NO predominantly resides in the lower energy orbitals of the ferrous ion (61% Fe ( $d_{xy}, d_{yz}$ ) : 25% NO( $\pi^*$ )), but irradiation with relatively long wavelength light (>390nm) imparts enough energy to transfer it to a higher energy orbital located on the NO group (23% Fe : 73% NO) (Wolfe & Swinehart, 1975; Leeuwenkamp et al, 1984). This transition to an excited state causes a weakening of the metal-ligand bond strength, and can result in bond cleavage as the bond disposes of its excess energy. This is

Figure 1:1

The structure of nitroprusside.

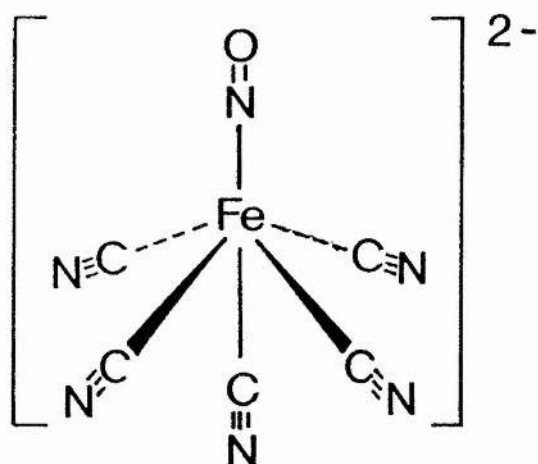


Table 1:1

Electronic spectrum of nitroprusside.

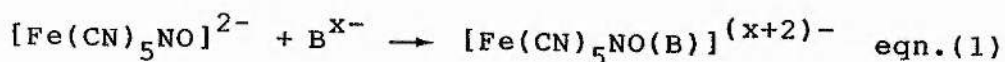
Band	$\lambda_{\max}$ (nm)	$\epsilon$ ( $M^{-1}cm^{-1}$ )	Assignment
I	498	8	$2b_2 \rightarrow 7e$ $d_{xy} \rightarrow \pi^*(NO)$
II	396	25	$6e \rightarrow 7e$ $d_{xy,yz} \rightarrow \pi^*(NO)$
III	330	40	$2b_2 \rightarrow 3b_1$ $d_{xy} \rightarrow d_{x^2-y^2}$
IV	265	900	$6e \rightarrow 5a_1$ $d_{xz,yz} \rightarrow d_z^2$
V	238	700	$6e \rightarrow 3b_1$ $d_{xz,yz} \rightarrow d_{x^2-y^2}$

(after Wolfe & Swinehart, 1975 and Leeuwenkamp et al, 1984)

summarised in table 1:1 which shows the electronic spectrum of NP together with the character of the absorption bands. Notice that wavelengths shorter than 400nm are all involved with d-d electron transitions. This would result in nonspecific metal-ligand cleavage (ie loss of  $\text{CN}^-$ ) and may account for the various photolytic products reported (ie. prussian blue, cyanide, ferrous ions etc) (Wolfe & Swinehart, 1975). A more extensive review of the photochemistry of NP is discussed in the introduction to chapter 4.

### iii. Chemical reactions

Nitroprusside has numerous reactions with a variety of inorganic and organic substances. Its propensity for forming highly coloured addition products has led to its use as a test reagent for numerous organic functional groups (for full list see Swinehart, 1967). In pharmaceutical analyses, these include the detection of ketones in urine of diabetics and of glutathione and cysteine in blood (Vesey & Batistoni, 1977). As already mentioned, the nitrosyl group is responsible for this reactivity as its formal charge of +1 means that it is open to attack from basic ligands (eg.  $-\text{OH}^-$ ,  $-\text{SH}^-$ ,  $\text{SO}_3^-$ ) (Swinehart, 1967; Leewenkamp et al, 1984; Butler & Glidewell, 1987). The addition reaction involved is shown in equation 1, where B= basic ligand.



In this regard, the chemical reaction of greatest physiological interest is that which occurs with thiols. Thiol groups have been implicated in the activation of guanylate cyclase by NP and other nitrovasodilators (Needleman & Johnson, 1973). It has even been suggested that formation of

## Chapter 1

S-nitroso-thiols is a prerequisite intermediate step in the mechanism of action of these vasodilators (Ignarro, Lippton, Edwards, Baricos, Hymen, Kadowitz & Gruetter, 1981). The reaction sequence that leads to the formation of nitrosothiols is as shown in equation 2. Thiol ligands (R-SH: where R represents an organic group) must first undergo deprotonation (to  $R-S^-$ ) before they can react with nitroprusside to form the addition product, except when RSH represents an amino thiol (eg. cysteine) when the protonated form has a similar rate of reaction (Butler & Glidewell, 1987). In the physiological pH range 6.5-8.5 this addition product loses the nitrosothiol ( $R-SNO$ ) to yield  $[Fe(CN)_5]^{3-}$  which may undergo a ligand redistribution to form  $[Fe(CN)_6]^{4-}$  and  $Fe^{2+}$  (Ignarro et al, 1981; Butler & Glidewell, 1987). The nitrosothiol in turn decays to nitric oxide (NO) and a disulphide ( $R-SS-R$ ). The addition step in this reaction proceeds slowly but once formed, the addition product decomposes quickly (Leewenkamp et al, 1984; Craven & Derubertis, 1978). An alternative pathway for the decomposition of the adduct has been reported to form  $[Fe(CN)_4NO]^{2-}$  and free cyanide (Butler & Glidewell, 1987). Which of these pathways is dominant in vivo has yet to be resolved. The latter scheme could account for the reported in vivo release of cyanide (Leewenkamp et al, 1984?), and the former one for the reported nitrosothiol and disulphide formation after NP administration (Ignarro et al, 1981; Needleman & Johnson, 1973).

### Clinical Uses

Ever since Page and colleagues (1955) demonstrated the

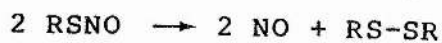
Table 1:2

Toxicity ( $LD_{50}$  values) of nitroprusside compared to cyanide and thiocyanate.

Species	Route of injection	LD <sub>50</sub>		
		mg/kg	mm/kg	CN mEq/kg
<u>Na<sub>3</sub>Fe(CN)<sub>5</sub>NO · 2 H<sub>2</sub>O</u>				
Mouse	i.p.	9.4	0.032	0.16
Mouse	i.p.	8.9	0.030	0.15
Mouse	i.p.	12.0	0.040	0.20
Mouse	i.v.	8.0	0.027	0.14
Rat	i.p.	9.9	0.033	0.17
Rat	i.v.	9.3	0.031	0.16
<u>NaCN</u>				
Mouse	i.p.	5.9	0.120	0.12
<u>KCN</u>				
Mouse	i.v.	2.6	0.040	0.04
<u>NaSCN</u>				
Mouse	i.v.	483.5	5.960	5.96
Rat	i.p.	540.0	6.660	6.66

(taken from Kreye, 1980).

Equation (2).



(after Butler & Glidewell, 1987 and Leeuwenkamp et al, 1984).

## Chapter 1

efficacy of intravenous infusion of NP in the treatment of malignant hypertension, it has been used in a variety of hypertensive conditions. Oral administration, previously used to treat arterial hypertension, was shown to be not nearly as effective and induce the same effects as sodium thiocyanate (Page et al, 1955). The major advantages of NP infusion are; (i) it has an immediate onset of action, (ii) the extent of the hypotensive response can be accurately controlled by the rate of infusion, (iii) there is a very rapid reversal of its effects when the infusion is stopped, (iv) There are no untoward interferences with any other drugs, (v) it does not develop primary resistance (tolerance) or tachyphylaxis (vi) it is still effective when other drugs fail to lower blood pressure, (vii) it has a selective action on the vasculature without any negative inotropic effect on the heart (Kreye, 1980).

The major disadvantages of NP are; (i) its potent hypotensive action requires careful and constant monitoring of the patient's blood pressure so that the infusion rate can be continuously altered to maintain the desired depressor effect, and (ii) its potential toxicity (this is discussed later). Too rapid infusions of NP are dangerous for two reasons. Firstly, because of its high potency, a rapid infusion or change in the patient's position can result in an exaggerated hypotensive response, with all the associated symptoms of circulatory failure. Secondly, excessive doses of NP can lead to cyanide poisoning. There have been a number of reported fatalities following excessive infusions of NP, all of which showed the classical signs of cyanide poisoning (Kreye, 1980). To prevent



this acute toxicity, it is generally agreed that a maximum rate of 800-900 ug/min. should not be exceeded. Concomitant infusion of cyanide antedotes has also been suggested. The potential toxicity of nitroprusside through release of its cyanide ligands is discussed later.

The conditions successfully treated by NP administration are:-

i. Hypertensive emergencies and malignant hypertension

There are numerous clinical conditions giving rise to a hypertensive crisis, most of which have been successfully treated by NP infusion. These range from hypertension associated with renal failure, pulmonary oedema, subdural or intracranial hemorrhage, pre- and intra-operative hypertensive attacks, and drug induced hypertensive crisis (Kreye, 1980). Some authors have recommended the use of NP as the primary agent in hypertensive emergencies with subsequent substitution by oral hypertensives, whereas others have kept NP 'in reserve' until other potentially less harmful drugs have been tried (Kreye, 1980).

NP has little role to play in the treatment of malignant hypertension in the ambulatory patient, but it has been successfully used to treat malignant hypertension by continuous infusion over periods up to 45 days (Page et al, 1955). Constant monitoring of plasma thiocyanate levels is required throughout. Thiocyanate is the final in vivo breakdown product of NP which although of low toxicity, is excreted very slowly such that toxic levels can be reached (chronic toxicity).

ii. Cardiac Failure



Irrespective of the underlying cause, cardiac failure is associated with symptoms of low cardiac output and/or circulatory congestion. Arterial vasodilators decrease afterload (ie. aortic or pulmonary impedance) and so increase cardiac output, whereas venodilators produce venous pooling and hence a reduction in preload, such that cardiac filling pressure is reduced. Since NP is a nonselective vasodilator, it induces both of the above effects and so is very successful in improving cardiac performance. As NP has to be infused, its administration is limited to the acute therapy of cardiac failure. It is reported to reduce left ventricular filling pressure and increase stroke volume in patients with acute myocardial infarction (Chatterjee, Swan, Kaushik, Jobin, Magnusson & Forrester, 1976). Although NP improves cardiac performance in acute myocardial infarction in the short term, it increases the extent of ischemic myocardial damage as the associated coronary vasodilation reduces perfusion pressure and induces 'stealing' of blood to unaffected areas. This is shown by an increase in the long term mortality rate of acute myocardial infarct patients following NP therapy (Chatterjee et al, 1976).

### iii. Controlled Hypotension in Surgery

The aforementioned properties of NP make it an ideal drug to induce hypotension during surgery under anaesthesia. This is applied to minimise blood loss during surgical procedures which prevents obscuring the field of operation, reduces the number of blood transfusions required (and so cuts down operating time and expense), and it reduces the probability of haemorrhage in delicate cerebral and intraocular

surgery (Kreye, 1980). These factors have been shown to decrease the mortality rate following surgery.

### Toxicity

The potential toxicity of NP is the major criticism of its clinical use today. There are numerous reports that NP releases cyanide when intravenously infused into either man or animals (Kreye, 1980; Arnold, Longnecker & Epstein, 1984; Smith & Kruszyna, 1974; Vesey & Batistoni, 1977; Page et al, 1955). There have been several fatalities following administration of higher than average doses of NP (Kreye, 1980), all of which display the classical signs of cyanide poisoning. At least three humans have successfully committed suicide using NP with cyanide being found in the stomach contents of all three (see refs in Kreye, 1980). In toxicity trials in mice and rats, intraperitoneal injection of NP was shown to have fourfold greater toxicity than  $\text{CN}^-$  alone (Smith & Kruszyna, 1974). This was taken as evidence that NP liberated all of its five cyanide groups in vivo. If injected intravenously, cyanide had a very similar lethal dose. This is summarised in figure 1:2 which shows the lethal doses ( $\text{LD}_{50}$ ) of NP given by either route to small rodents.

The better invasion and distribution properties of the i.v. route of administration would seem to refute the earlier suggestion that NP releases all its cyanide groups. Comparing the substances with regard to their cyanide content, it appears that NP is four fold less toxic than cyanide alone, with only one cyanide group being released per molecule of NP. The time

delay between application and death is very much longer for NP than cyanide. Intravenous administration of KCN caused death within 2 minutes but it took 15 min. after NP administration. Intraperitoneal applications took longer with death occurring after 5min with KCN but as long as 30min. with NP. The longer time for the toxic effect of NP must be involved with the reactions that release cyanide ligands. This is in contrast to its fast vasopressor action which occurs instantaneously with i.v. administration.

Detoxification of cyanide is performed through an enzymic reaction catalysed by rhodanase. This system converts cyanide to thiocyanide which is 150 times less toxic (see table 1:2). The high activity of this enzyme in the liver and other tissues ensures that the body can inactivate quite large concentrations of cyanide, the limiting factor being the availability of thiosulphate. Thiosulphate is therefore used as an antidote to cyanide poisoning and has been used to counteract signs of cyanide toxicity in patients receiving NP infusions. Although of low toxicity, thiocyanate is excreted very slowly ( $t_{1/2} = 8$  days) such that its blood level must be constantly monitored to prevent toxic levels being reached during long term NP infusions (Page et al, 1955).

#### **Mechanism of cyanide release from NP**

The mechanism of cyanide release has mainly been studied in vitro by incubating NP with different tissue homogenates. Liver and kidney were found to be slightly active but heart, brain, small intestine, skeletal muscle and lung homogenates were not (Smith & Kruszyna, 1974). Blood was by

far the most active tissue tested, with the order of cyanide release being red cell lysates > whole blood > washed red cells > plasma (Page et al, 1955; Smith & Kruszyna, 1974). The observation that protein free filtrates of blood can similarly liberate cyanide over wide ranges of pH and temperature suggested that the reaction was non-enzymatic (Page et al, 1955). This is substantiated by the ineffectiveness of enzyme inhibitors to prevent liberation of cyanide from NP in the presence of whole blood (Speigel & Kucera, 1977). As NP is known to react with sulphydryl groups (see above) it seemed likely that sulphydryl containing free amino acids in the blood might be responsible for this action. Incubation of some of these compounds (namely cysteine and glutathione) with NP liberated cyanide (Page et al, 1955). The rich sulphydryl content of erythrocytes, along with the high glutathione content of liver and kidney, support this hypothesis.

Smith & Kruszyna (1974) went on to suggest that a secondary fast reaction with haemoglobin could explain the high rates of cyanide release seen with red cell lysates or whole blood. In this reaction, NP receives an electron from haemoglobin to form methaemoglobin (metHb). The reduced NP then decomposes quickly to yield five cyanide groups, one of which reacts with metHb to form cyanohaemoglobin. It is however unlikely that this reaction has any physiological significance to the in vivo degradation of NP because it is unable to penetrate the erythrocyte membrane at therapeutic concentrations (Rodkey & Collison, 1977). NP is similar in structure to ferricyanide which is known not to penetrate intact cell membrane (Gruetter, Gruetter, Lyon, Kadowitz &

Ignarro, 1981). It has been proposed that species differences in the ability of whole blood to breakdown NP is related to differing permeabilities to NP (Smith & Kruszyna, 1974). Rodkey & Collison (1977) found however that 90% of NP was present exclusively in the plasma. After a bolus injection of NP into dogs, the peak level of cyanide in the plasma precedes that in the erythrocyte (Kreye, 1980). Taken together, this is evidence that the decomposition of NP occurs outside the erythrocyte without involving Hb. If any cyanide is liberated, it would be rapidly 'mopped' up by the Hb, which accounts for the observation that 90% of the released cyanide is present inside erythrocytes (Kreye, 1980). Incidentally, trapped cyanide would have little toxicological significance because free cyanide in the plasma is the critical factor in determining the acute toxicity of NP (Kreye, 1980).

A major criticism of the above work is that none of the studies mention (and so presumably overlooked) the precautions taken to protect NP from light during both the incubation and analysis for cyanide. This has important implications as NP is extremely photolabile and its photolytic product, aquapentacyanoferrate (Aq), more readily releases cyanide, especially at low pH. Since the analytical procedure normally used to determine cyanide involves acidifying the sample, then it will serve to liberate  $\text{CN}^-$  from any Aq present. The amount of HCN measured will be the total cyanide present both free and that bound to Aq. Bisset, Butler & Glidewell (1981) have reported no release of cyanide from NP incubated with whole blood, plasma or erythrocytes, if light is rigourously excluded at all stages of the experiment and

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analysis. Conversely, if the analysis is conducted in light, then cyanide liberation can be demonstrated. Preformed Aq subjected to the analysis also liberates cyanide whether illuminated or not (Butler & Glidewell, 1987). Subsequent studies using nuclear magnetic resonance (NMR) to follow the fate of NP labelled with  $^{13}\text{C}$ , showed no trace of cyanide liberation when NP was incubated in the dark with whole blood (Butler, Glidewell, 1987). The in vivo release of cyanide has also been questioned by these authors. If NP was illuminated before or during infusion, any Aq formed would liberate  $\text{CN}^-$  in vivo as the pH of blood is lower than the pK for HCN formation (Bisset, Butler & Glidewell, 1981). Also, any analysis for cyanide undertaken on a blood sample which contains NP in the light, will measure cyanide released through artefactual photolysis during the analysis (which takes 2-3 hours) and not that actually released in vivo. In view of this, all results reporting cyanide release either in vitro or in vivo must be interpreted cautiously.

Is in vitro degradation of NP similar to the in vivo situation ?

Only decomposition studies conducted in complete darkness give a realistic measure of cyanide release that is devoid of any artefactual photolysis. Arnold, Longnecker & Epstein (1984) still found in vivo release of cyanide even though NP was protected from light during infusion into patients and during blood sample analysis for cyanide. Leeuwenkamp et al (1984) used a sensitive polarographic technique to measure the in vitro degradation of NP in the presence of various blood components in the dark. Although



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degradation was observed with all blood components, the amount and rate of degradation were far too small to account for the in vivo situation. The rapid restoration of blood pressure after cessation of NP infusion indicates a fast in vivo conversion. Whereas incubation with whole blood results in destruction of NP with a half life of 20-60 minutes, the in vivo half life is 30-120 sec (Smith & Kruszyna, 1974; Kreye, 1980; Leeuwenkamp, Chin, Van der Mark, Bennekom & Bult, 1986). Similarly, degradation by this mechanism could not be involved in the mechanism of action of NP. Page et al (1955) admitted that the rate of decomposition by blood components is too slow for it to be an essential step in its depressor activity. The long incubation times required for in vitro degradation may be a consequence of photolysis being primarily responsible. It is clear that degradation of NP by blood or any of its components is not associated with the in vivo situation.

A clue as to the nature of the in vivo degradation came from the observation that 30-35% of NP was destroyed during a single passage through the perfused hind limb of the rat (Kreye, 1980). Incubation with crude aortic soluble fraction in the dark resulted in rapid breakdown of NP, at a rate comparable to in vivo degradation rates (Leeuwenkamp et al, 1986). It would appear from this that vascular smooth muscle is responsible for the in vivo decomposition. As NP acts directly on the vasculature, its site of action and degradation are the same. Indeed, the destruction of NP is thought to be essential for its vasodilator action.

It should be noted that in the study by Leeuwenkamp et al (1986), the change in the polarographic signal generated by

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NP was used to determine its degradation. Consequently, the nature of the chemical change is not defined, but could result from a loss of the nitrosyl moiety, as opposed to cyanide ligand. As most of the other studies are concerned with the latter reaction, they have attempted to measure cyanide release and not the amount of intact NP remaining. This may be another factor why the degradation rate is so much faster, as this technique records the primary reaction liberating NO, and not the subsequent ones releasing cyanide. This also has important implications as to the mechanism of degradation especially since it seems to be associated with the vasopressor action.

### Cellular mechanism of action of NP

It is well documented that NP enhances the cyclic guanosine-3'-5'-monophosphate (cGMP) concentration in a wide variety of tissues. Cyclic GMP has been implicated as an intracellular regulator of cellular function in many tissues, because the rise in cGMP induced by NP is associated with the tissue response (eg. vascular smooth muscle relaxation). Nitroprusside is thought to elevate cGMP levels by directly stimulating the activity of guanylate cyclase (GC), the enzyme that catalyses the formation of cGMP from GTP (guanosine triphosphate). This enzyme is present in virtually all cell types and phyla, although it is only recently that its important cellular function is becoming recognised. It exists in two different forms, soluble (cytosolic) and particulate (membrane bound), although isoenzymes of each type with different subcellular locations have been reported (Waldmann &



Murad, 1987). Although NP is reported to stimulate both types of GC, there is now doubt as to whether it can activate the particulate form. Purified particulate GC is usually contaminated with soluble GC, and if this is removed by successive hypo- and hypertonic washes, the activation by NP is virtually abolished (reduced to 1% of its former activation) (Waldman & Murad, 1987). Highly purified particulate GC cannot be activated by NP, although the use of detergents in the purification procedure confuse this issue as they have also been shown to prevent NP stimulation of soluble GC (Waldman & Murad, 1987). Soluble GC is the better understood enzyme as it is easier to purify and is more homogeneous than its particulate counterpart. Enzyme isolated from a wide variety of tissues has been shown to be markedly activated by NP (Arnold, Mittal, Katsuki & Murad, 1977; Waldman & Murad, 1987). The activation of soluble GC to increase cGMP levels is the fundamental step in the mechanism of action of NP and so this section will concentrate on the various steps involved. Attention will be focused only on soluble GC, and especially that associated with vascular smooth muscle.

#### **Nitric oxide release from 'nitrovasodilators'.**

The nitrovasodilators are a group of hypotensive agents that possess, generate or release nitric oxide. This diverse group includes inorganic and organic nitroso compounds (eg. NP,  $\text{NaNO}_2$ ), organic nitrates (eg. glyceryltrinitrate (GTN), nitrosoguanidine) and nitrate esters. Since all of these compounds can lead to the formation of nitric oxide, and since they all relax vascular smooth muscle and stimulate

isolated GC, it was suggested that nitric oxide was a common intermediate in their mechanism of action (Ignarro & Kadowitz, 1985; Murad, Arnold, Mittal & Braughler, 1979; Ignarro, Lipton, Edwards, Baricos, Hymen, Kadowitz & Gruetter, 1981; Gruetter, Barry, McNamara, Kadowitz & Ignarro, 1980; Waldman & Murad, 1987). Evidence for this is that dissolved nitric oxide evokes similar relaxation and cGMP accumulation in coronary smooth muscle as that produced by NP and other nitrovasodilators (Gruetter et al, 1980). The protein phosphorylation profile which is associated with relaxation of vascular smooth muscle is the same after NP, 8-bromo-cGMP or nitric oxide treatment suggesting an identical mechanism of action (Rapoport, Draznin & Murad, 1982). Studies with purified soluble GC have shown a direct stimulatory effect of nitric oxide, the size of which is similar to that produced by nitrovasodilators (Arnold, Mittal, Katsuki & Murad, 1977). Activation of soluble GC with nitric oxide is not additive to that by other nitrovasodilators, which again tends to support the hypothesis that they act through formation of nitric oxide (Waldman & Murad, 1987).

As nitric oxide is the prerequisite intermediate, the efficacy of the nitrovasodilators is dependent on their ability to generate it (Ignarro et al, 1981; Waldman & Murad, 1987; Ignarro & Kadowitz, 1985; Schroeder, Noak & Muller, 1985). The activation of isolated liver GC by simple nitrovasodilators, azide and hydroxylamine, is lost upon further purification because they depend on the presence of a cofactor (probably catalase) that catalyses their oxygen dependent conversion to nitric oxide (Waldman & Murad, 1987).

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Other studies suggest that isolated GC from tissue that is unresponsive to these agents (ie heart & lung), contains an inhibitor (probably haemoglobin or myoglobin) that prevents activation, by binding nitric oxide or preventing its formation (Kimura, Mittal & Murad, 1975).

Larger nitric oxide containing nitrovasodilators must first liberate their nitric oxide moiety before they can relax vascular smooth muscle or stimulate GC (Brien, McLaughlin, Breedon, Bennet, Nakatsu & Marks, 1986; Brien, McLaughlin, Kobus, Kawamoto, Nakatsu & Marks, 1987; Schroeder, Noak & Muller, 1985). Measurements of the biotransformation of GTN to GDN correlate well with the increased cGMP level and induced relaxation of rabbit aortic strips (Brien et al, 1986). Evidence that this is a causal relationship comes from an elegant time course study following the degradation of tritium labelled GTN, which showed that denitration of GTN preceded both relaxation and the rise in tissue [cGMP] (Brien et al, 1987). Similarly, the activity of isolated GC stimulated by GTN and other organic nitrates parallels the amount of nitric oxide liberated (Schroeder et al, 1985). Interestingly, only one molecule of nitric oxide is released from each drug molecule even if it contains several nitrate residues (ie. GTN). Nitroprusside was previously thought to 'spontaneously release nitric oxide in solution' (Ignarro et al, 1981; Ignarro & Kadowitz, 1985; Waldman & Murad, 1987), but this would appear to be due to artefactual photolysis as solutions of NP are completely stable if light is rigourously excluded (Vesey & Batistoni, 1977; Arnold, Longnecker & Epstein, 1984; Butler & Glidewell, 1987). A recent study in which NP was

completely protected from light throughout showed that it does release NO when incubated with bovine aortic soluble fraction in a similar manner to other nitrovasodilators (Leeuwenkamp et al, 1986).

The questions then arise, how is the nitric oxide intermediate liberated from the parent molecule, and how does it stimulate GC activity? Although various agents have been postulated to mediate this cleavage, there are now two major schools of thought. The first is that free thiols in the cell membrane or cytosol (probably cysteine) combine with the nitrosyl group to form unstable nitrosothiol intermediates which then stimulate GC (Ignarro et al, 1981; Ignarro & Kadowitz, 1985; Schroeder et al, 1985). The second school postulates that the precursor drug interacts directly with a prosthetic haem group associated with the GC which binds the nitric oxide and results in increased activity of the enzyme (Craven & DeRubertis, 1983). The evidence supporting each hypothesis will now be reviewed separately.

#### Role of thiols in GC activation

An early indication that sulphydryl(thiol) groups were involved came from the observation that high doses of organic nitrate (GTN) can induce cross-tolerance to subsequent applications of other organic nitrates but not to other types of vasodilator (Needleman & Johnson, 1973). This suggested that the organic nitrates themselves changed the availability of a common intermediate 'receptor'. These were identified as sulphydryl groups because; (i) treatment with the -SH alkylating agent ethacrynic acid, which reduced the measureable tissue sulphydryl content, caused a decrease in

the magnitude of relaxation induced by GTN; (ii) pretreatment with large doses of GTN, sufficient to cause tolerance, also reduced the tissue -SH content; (iii) disulphide reducing agents (ie. dithiothreitol) could prevent or reverse tolerance, regardless of whether it was induced in vivo or in vitro; (iv) other known -SH oxidizers are also potent vasodilators (eg. NP, cystamine) and cross tolerance to these can be induced by pretreatment with GTN (Needleman, Jakschik & Johnson, 1973; Needleman & Johnson, 1973). The above results led to a reaction scheme in which organic nitrates and other vasodilators, could only react with reduced sulphydryl groups (R-SH) in vascular smooth muscle, resulting in their oxidation to form disulphides (R-S-S-R). This step was coupled to release of inorganic nitrite which stimulated guanylate cyclase (GC) (Needleman et al, 1973). This scheme incorporates some of the known sulphydryl oxidising properties of NP, described earlier (Butler & Glidewell, 1987). The release of 'nitrite' is now thought to result from an actual release of nitric oxide (NO) which then combines with molecular oxygen. Indeed, the liberation of NO has been shown to occur if GTN, NP or  $\text{NaNO}_2$  are incubated with thiols under certain conditions (Ignarro & Kadowitz, 1985). It is the liberation of nitric oxide (NO) that is thought to be responsible for GC activation (Ignarro et al, 1981; Waldman & Murad, 1987; Ignarro & Kadowitz, 1985)

Desensitisation can also be seen at the level of the partially purified guanylate cyclase enzyme (Waldman, Rapoport, Ginsberg & Murad, 1986). Soluble guanylate cyclase prepared from vascular smooth muscle previously rendered

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tolerant to GTN, has a diminished response to NP, GTN, and NO as compared to normal control enzyme (Waldman et al, 1986). This phenomenon could involve sulphydryl groups on the enzyme itself which play a key role in regulating its activity (see later) (Waldman & Murad, 1987).

Direct evidence for a role of thiols has come from investigations into the activation of isolated GC by various nitrovasodilators. The activation of unpurified coronary arterial GC by NO, NP and other nitrovasodilators was enhanced by the addition of thiols, the most potent being cysteine (Gruetter, Barry, McNamara, Kadowitz & Ignarro, 1980). Indeed, GTN failed to activate GC except when the thiol cysteine was present (Ignarro & Gruetter, 1980). The degradation of GTN was measured during activation of GC in the presence of various thiols (Schroder, Noak & Muller, 1985). Only in the case of cysteine was there a quantitative correlation between the amount of NO liberated and the extent of GC activation (Schroder et al, 1985). The lack of a correlation between the (high) degradation rate and the (low) stimulatory potency of other thiols, has led to the suggestion that thiols may modulate GC activation in some other way beside NO release (Schroder et al, 1985).

The observation that nitrovasodilators react with thiols to form unstable S-nitrosothiol compounds, has led to the view that these substances are intermediates in the activation of GC (Ignarro, Lipton, Edwards, Baricos, Hyman, Kadowitz & Gruetter, 1981). The evidence for this is that; (i) although nitrovasodilators elevate tissue cGMP levels, they require the addition of cysteine to maximally activate



partially purified GC; (ii) all nitrovasodilators, including NO, react with cysteine to form S-nitrosocysteine; (iii) preprepared S-nitrosothiols are approximately 100 times more potent at activating partially purified GC than nitrovasodilators (NP) alone (iv) preformed nitrosocysteine markedly activates GC, raises tissue cGMP levels, relaxes coronary arterial strips and decreases systemic blood pressure, similar to those effects of nitrovasodilators; (v) methylene blue and ethacrynic acid, which are known to inhibit nitrovasodilator induced coronary arterial relaxation and cGMP accumulation, also inhibit the effects of S-nitrosothiols; (vi) when infused into anaesthetised cats, preprepared S-nitrosothiols mimic the dose related effects of NP and GTN (Ignarro et al, 1979-1981; Gruetter et al, 1980; Ignarro & Gruetter, 1980; Ignarro & Kadowitz, 1985). The clinical effects of nitroglycerin in reducing arterial pressure are potentiated by preinfusion of acetylcysteine, which also served to reduce tolerance to GTN (Horowitz, Antman, Lorell, Barry & Smith, 1983). From this evidence, it was proposed that the nitrogen oxide containing vasodilators caused vascular smooth muscle relaxation by interaction with intracellular thiols (primarily cysteine) to form S-nitrosothiols which then activate GC, resulting in cGMP formation (Ignarro et al, 1981). The above argument suggests that thiols are responsible for cleaving the NO moiety off the parent nitrovasodilator molecule. This could explain the dependency of drug action on thiol groups (Needleman & Johnson, 1973), as their availability might then determine the amount of active S-nitrosothiol formed.

The involvement of nitrosothiols in directly activating GC has been criticised by Craven & DeRubertis (1983), who suggest that GC activation occurs instead through the formation of a NO-haem complex without any involvement of thiols. When GC is purified to apparent homogeneity, so that the enzyme lost the associated haem group, it does not respond to NO, NP or preformed nitrosocysteine. By contrast, preformed NO-haem markedly (60-80 fold) increases purified enzyme activity (Craven & DeRubertis, 1983). Addition of exogenous hematin restores the ability of all the agents to activate the enzyme. Also, at neutral pH, S-nitrosothiol formation cannot be detected between NP and cysteine (Craven & DeRubertis, 1983). Preformed nitrosocysteine decomposes rapidly, but even fully decomposed solutions retain their ability to activate GC providing haem is present (Craven & DeRubertis, 1983). The reported activation of haem-containing GC by thiols, is suggested to be due to the reducing action of thiols facilitating the formation of NO-haem by maintaining haem iron in its ferrous ( $\text{Fe}^{\text{II}}$ ) state (Craven & DeRubertis, 1983; Waldman & Murad, 1987). These observations suggest that while S-nitrosothiols may activate haem-containing GC, they are not obligatory intermediates in the activation process and that the nitrosyl-haem group acts in this capacity.

#### **Role of the prosthetic haem group of guanylate cyclase**

An early indication that haem groups could have a profound effect on the activity of GC was that simple nitrovasodilators (ie. azide) required the presence of a haemoprotein before they could activate the enzyme (Kimura et



al, 1975; Waldman & Murad, 1987). Greater importance came from the observation that activation of crude isolated GC by NO and by nitrovasodilators was lost upon further purification of the enzyme (Murad, Lewicki, Brandwein, Mittal & Waldman, 1981). Activation by these agents could be restored by the addition of haemoglobin, methaemoglobin or catalase (Craven & DeRubertis, 1978). The activation was enhanced by including a reducing agent into the incubation medium which facilitates the formation of nitrosyl haem from haemoproteins and NP or NO (Craven & DeRubertis, 1978). Subsequent authors using improved purification techniques, managed to purify GC without loss of stimulation by NP, and showed that this was due to the presence of a detectable prosthetic haem group associated with the enzyme (Gerzer, Hofmann, Bohme, Ivanova, Spies & Schultz, 1981). Whether the haem group is a normal prosthetic group for the enzyme in vivo or whether their association is an artefact of preparation remains unknown and is still a matter of controversy (Waldman & Murad, 1987).

The importance of the prosthetic haem group in the activation of GC by nitrovasodilators is highlighted by studies with haem deficient enzyme. The purified enzyme is totally unresponsive to NO, NP or preformed nitrosothiols but can be maximally activated by preformed nitrosyl haemoglobin (Craven & DeRubertis, 1983). Preformed NO-haem is 10 fold more potent at activating GC than nitro compounds or their derivatives, even in the presence of haem. The observation that reducing agents could potentiate nitrovasodilator activation but not that due to preformed NO-haem, supported the suggestion that such agents serve only to enhance the

formation of NO-haem from the nitrocompounds (Craven & DeRubertis, 1983). Agents which bind to haem groups (eg. cyanide) block activation of GC by azide, NP or NO (Kimura, Mittal & Murad, 1975; Rapoport & Murad, 1984). Cyanide also abolishes the NP and NO induced relaxation and cGMP accumulation in rat aortic strip, but not those induced by 8-bromo-cGMP (Rapoport & Murad, 1984). The above evidence supports the hypothesis that activation of soluble GC by nitrocompounds involves the interaction of NO with a haem moiety associated with the enzyme (Waldman & Murad, 1987).

The observation that mixtures of NP and haemoglobin in the presence of a reducing agent at pH 7.6, readily form NO-Hb has led to the suggestion that nitrovasodilators interact directly with the haem group of GC to form nitrosyl-haem (Craven & DeRubertis, 1983). The activation of GC by preformed NO-Hb supports this hypothesis. Also, the release of nitric oxide from GTN in the presence of thiols is markedly enhanced (10-15%) by the addition of GC to the incubation medium (Schroder et al, 1985). This evidence suggests that GC is directly involved in nitro-compound cleavage. Measurements of the degradation of NP by crude aortic soluble fraction, in the dark and in the presence of excess cyanide, contradict this hypothesis (Leeuwenkamp et al, 1986). As cyanide has a high affinity for haem, it should bind to the haem group and prevent it reacting with the NO moiety on NP (leeuwenkamp et al, 1986). Instead, excess cyanide did not affect the rate of NP degradation suggesting that it decomposes due to interaction with a reductor other than the haem group of GC (Leeuwenkamp et al, 1986).

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Direct interaction of NP with GC would imply an intracellular site of degradation and action. Evidence for this is that NO induced coronary arterial relaxation is inhibited by methylene blue, but not by the impermeant ferricyanide although both compounds inhibit isolated soluble GC (Gruetter et al, 1979,1980; Arnold et al, 1980). Impermeant hemoproteins (haemoglobin & methaemoglobin) can abolish relaxations induced by NO but not by any of the nitrovasodilators (Gruetter et al, 1979). From this, Ignarro (1981) has suggested that the efficacy of the various nitrovasodilators is related to their ability to cross membranes. While this may be true for the lipophilic nitrovasodilators (ie.NO, amyl nitrate, GTN), it becomes a problem for the impermeant ones, especially NP. The high potency of NP does not correlate well with its low permeability. Explanations that NP spontaneously releases NO, may be a consequence of artefactual photolysis (Ignarro et al, 1981; Ignarro & Kadowitz, 1987). A possible alternative is that NP could interact with thiol groups in the cell membrane to liberate NO. The test for this, measuring the degradation of NP when incubated with sarcolemmal fraction from vascular smooth muscle does not appear to have been done.

Taking an overview of all the above evidence, the most likely sequence of events in nitrocompound activation of GC is; (i) the drug molecule interacts with thiols either in the sarcolemma or cytosol of the vascular smooth muscle cell, which cleave the NO group off the drug, (ii) The liberated NO and thiol groups form transient unstable nitrosothiols, (iii) the nitrosothiols degrade yielding the NO to the haem group

associated with GC (iv) the nitrosyl-haem group activates GC to increase production of cGMP.

#### Redox regulation of guanylate cyclase

From the above evidence, it is clear that the activation of GC by nitric oxide and related nitrocompounds, involves several factors that are vulnerable to oxidation-reduction agents. These include; (i) the tissue thiol groups must be in the reduced state before they can promote the liberation of NO from nitrocompounds or form nitrosothiols, (ii) the formation of nitrosyl-haem responsible for activation of GC is enhanced if haem iron is present in its reduced ( $\text{Fe}^{\text{II}}$ ) state, (iii) Oxidising agents promote the conversion of NO to higher oxides which do not stimulate GC. These are responsible for the observed decrease in the nitric oxide induced activation of GC in liver supernatants when incubated in a high oxygen environment (Arnold, Mittal, Katsuki & Murad, 1977). Similarly, the activation by NO can be inhibited by oxidising agents ( $\text{H}_2\text{O}_2$ , methylene blue,  $\text{K}_3\text{Fe}(\text{CN})_6$ ) whereas it is augmented by various reducing agents (ascorbate, cysteine, glutathione, dithiothreitol (DTT)) (Arnold et al, 1977). That some of these reducing agents are thiols suggests an additional role in their augmentation of activation by NO and the nitrovasodilators. Direct evidence for this is the lack of a correlation between the rate of GTN cleavage by various thiols, and their stimulatory potency on GC (Schroder, Noak & Muller, 1985). Cysteine had by far the greatest stimulatory effect on GC activity, despite liberating the same amount of nitric oxide as other thiols. This suggests that thiols act directly in modulating GC activation as well

as being a cofactor in organic nitrate cleavage (Schroder et al, 1985). Their relative potencies is thought to be due to their differing redox potentials (Schroder et al, 1985).

The oxidation state of purified guanylate cyclase has a profound effect on its activity. Incubation of purified enzyme in air or oxygen solutions resulted in an increase in activity (Haddox, Stephenson, Moser & Goldberg, 1978; Murad, Lewicki, Brandwein, Mittal & Waldman, 1981). Preincubations in an oxygen atmosphere for up to 20min (37°C) resulted in progressively increased GC activity (White, Crawford, Patt & Lad, 1976). However, this activity is reduced upon longer preincubations (>20min at 37°C; >30min at 30°C), a process that can be prevented or reversed by adding thiols (White et al, 1976). Enzyme activation followed by inactivation is very similar to that produced by nitric oxide activation of purified GC (Murad et al, 1981). Activation of enzyme with NO spontaneously deactivates, even in the presence of NO (Braugher, Mittal & Murad, 1979). Addition of DTT or other reducing agents, reverses this process and restores maximal activity.

There is an optimum concentration of NO, such that higher or lower concentrations do not activate the enzyme as effectively, giving rise to a bell-shaped dose response curve (Braugher et al, 1979). This curve is shifted to the left with purification of the enzyme and is altered by the addition of various proteins, thiols and sugars (Murad et al, 1981). The oxidant dehydroascorbic acid increases the activity of both isolated and cellular GC, whereas DTT or cysteine decreases it (Haddox, Stephenson, Moser & Godberg, 1978). DTT

and other reducing agents prevent and reverse activation of GC by either preincubation in an oxygen environment or activation by NO (Waldman & Murad, 1987). This contrasts with the well known effect of reducing agents in augmenting GC activation by NO and nitrocompounds (Waldman & Murad, 1987). The interpretation of these results is that oxidation of GC (by air, oxygen, nitric oxide or oxidants) initially results in activation, but the enzyme can be overoxidised (by longer incubations, high [NO] or long preincubations in oxygen) which leads to a decrease in activity. Reducing agents can decrease activity by preventing or reversing the initial oxidation. However, they can also increase activity by preventing or reversing overoxidation of the enzyme and by maintaining the enzyme in the reduced state such that the initial oxidising effect is greater. It is clear that the regulation of GC activity by redox agents depends on the previous oxidation state of the enzyme, and that there is an optimum 'oxidation state' for maximal activity. A complication is that most of these studies are performed on purified GC, which would be in totally different oxidation states depending on the purification procedures used, previous oxidation state in the tissue, and the incubation conditions of the enzyme assay. For these reasons it is hard to draw any conclusions about the in vivo situation from in vitro studies.

The targets for the redox regulation of GC have been suggested to be critical sulphhydryl groups on the enzyme (Waldman & Murad, 1987). Agents which modify free sulphhydryl groups alter basal and activated GC activity. Alkylating agents, which covalently modify -SH groups, decrease basal and



NO stimulated enzyme activity (Waldman & Murad, 1987). Mixed disulphide formation by incubating with cystamine or cystine, also reduces GC activity (Brandwein, Lewicki, & Murad, 1981; Murad et al, 1981). Radiolabelled [<sup>35</sup>S]cystine showed that radioactivity was incorporated into GC with a similar time course as inhibition of activity (Brandwein et al, 1981). DTT which reduces disulphides back to free thiols, reversed the inhibition of activity and this corresponded to a release of radioactivity from the enzyme (Brandwein et al, 1981). From this it was suggested that sulphhydryl groups on the enzyme are critical for basal and stimulated GC activity, and that thiol-disulphide transitions through redox reactions are an important mechanism for regulating GC activity.

#### **Nitrovasodilator induced increase in cGMP and relaxation**

There is now a wealth of evidence that cGMP is directly involved in mediating relaxation of vascular smooth muscle. The initial observations that led to the above hypothesis were that nitrovasodilator compounds induced relaxation and increased the cGMP levels in a variety of smooth muscle preparations (Kimura, Mittal & Murad, 1975; see Rapoport & Murad, 1983; Ignarro & Kadowitz, 1985). The increase in [cGMP] was dose dependent and correlated with the degree of relaxation induced by these compounds (Gruetter et al, 1979, 1980, 1981; Napoli, Gruetter, Ignarro & Kadowitz, 1979; Lincoln, 1983; Ignarro, Burke, Wood, Wolin & Kadowitz, 1983; see Rapoport & Murad, 1983). Evidence for a cause and effect relationship came from time course studies which revealed that the increase in [cGMP] induced by these agents precedes relaxation (Gruetter, Gruetter Lyon, Kadowitz &

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Ignarro, 1981; Ignarro, Lipton, Edwards, Baricos, Hyman, Kadowitz & Gruetter, 1981; Brien, McLaughlin, Kobus, Kawamoto, Nakatsu & Marks, 1987). Direct evidence that cGMP mediates relaxation is that lipid soluble analogues of cGMP (8-bromo-cGMP & dibutryl-cGMP) induce relaxation of precontracted arterial strips (Napoli et al, 1979; Lincoln, 1983; Rashatwar, Cornwell & Lincoln, 1987). Similarly, cGMP phosphodiesterase inhibitors raise intracellular [cGMP] and induce relaxation in coronary and aortic strips (Schoeffer, Lugnier, Demesy-Waeldele & Stoclet, 1987). Where they have been measured, cAMP levels or cAMP protein kinase activity appears to be unaltered by nitrovasodilators (Lincoln, 1983; Rapoport & Murad, 1983). In vascular preparations, nitrovasodilator effects were shown to be independent of endothelium, although in endothelial denuded preparations, they cause a greater increase in cGMP due to the lower basal control levels (Rapoport & Murad, 1983).

Various in vitro inhibitors of guanylate cyclase (GC) activity have proved useful in testing the hypothesis that nitrovasodilator induced relaxation is mediated through an increase in cGMP formation. Whereas lipophilic GC inhibitors (ie. methylene blue, cyanide, cystamine) inhibit relaxation and cGMP accumulation of arterial strips by all nitrovasodilators, impermeant ones only abolished the effects of free nitric oxide (haemoproteins) or had no effect at all (ferricyanide) (Gruetter, Lyon, Kadowitz & Ignarro, 1981; Rapoport, Brandwein & Murad, 1981). This suggested an intracellular site for liberation of NO.

The induction of tolerance also provides a test that



GC activation mediates relaxation in vivo. Nitroprusside induced relaxation and cGMP accumulation in rat aorta were inhibited by GTN pretreatment, either in vitro or in vivo (Waldman, Rapoport, Ginsberg & Murad, 1986; Rapoport & Murad, 1983). Purified GC, isolated from rat aorta made tolerant by GTN pretreatment, exhibited a marked desensitisation to activation by GTN, NP or NO, although its activation by other agents was not affected (arachidonic acid) or was increased (protoporphyrin IX) (Waldman, Rapoport, Ginsberg & Murad, 1986). This is strong evidence that nitrovasodilators all act through a final common pathway and that this involves activation of GC.

#### Endogenous nitrovasodilators

The above results indicate that cGMP mediates relaxation induced by exogenous agents (ie nitrovasodilators), but it has also been implicated in relaxation by endogenous agents (ie, acetylcholine, ATP, ADP, adenosine, autacoids, fatty acids, thrombin, substance P, vasopressin, histamine & bradykinin) (Rapoport & Murad, 1983). All of these agents can only relax vascular smooth muscle in the presence of an intact endothelium and so have been called 'endothelial dependent vasodilators' (Furchgott, 1983). In 1980, Furchgott & Zawadzki demonstrated that relaxation of a rabbit aortic strip by ACh was dependent on the release of an Endothelial Derived Relaxant Factor (EDRF). The release of EDRF has subsequently been shown to occur for all of the endothelial dependent vasodilators (Furchgott, 1983; 1987) Although its chemical identity was unknown, EDRF was shown to be the same substance by the use of

specific inhibitors (Furchgott, 1983). As the Ca ionophore A23187 is also an endothelial dependent vasodilator, release of EDRF was thought to be associated with an increase in endothelial [Ca] (Furchgott, 1981; 1983).

Studies of the mechanism of action of EDRF showed that it is very similar to that of the nitrovasodilators. EDRF increased intracellular cGMP levels in a dose dependent manner that preceded relaxation (Rapoport & Murad, 1983; Rapoport, Draznin & Murad, 1983; Ignarro, Burke, Wood, Wolin & Kadowitz, 1984; Furchgott 1987). Both EDRF-induced relaxations and increases in cGMP are inhibited by haemoglobin and methylene blue (Ignarro, Harbison, Wood & Kadowitz, 1985; Martin, Villani, Jothianandan & Furchgott, 1985). A specific cGMP phosphodiesterase inhibitor (M&B22948) enhanced EDRF-induced relaxation and increases in tissue cGMP (Martin et al, 1985). Basal release of EDRF was shown to occur, as haemoglobin and methylene blue increased smooth muscle tone whereas M&B 22948 decreased it (Martin, Furchgott, Villani & Jothianandan, 1986). From this it was suggested that EDRF induces vascular smooth muscle relaxation by directly stimulating GC to increase intracellular [cGMP] in an identical manner to the nitrovasodilators. This was substantiated by recent reports showing that pretreatment of rat aorta with GTN inhibits relaxation and cGMP accumulation by ACH (Rapoport, Waldman, Ginsberg, Molina & Murad, 1987). Cross-tolerance between endothelium dependent and independent vasodilators is strong evidence that they share a common mechanism of action.

The chemical identity of EDRF eluded investigators for several years and has only recently been discovered. Bioassays

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in which the endothelial release of EDRF (donor) was separated from an endothelial denuded smooth muscle strip (detector), revealed that EDRF is very labile, with a half life in Kreb's solution of between 6 and 50 seconds (Griffith, Edwards, Lewis, Newby & Henderson, 1984; Angus & Cocks, 1987; Furchgott, 1987; Moncada, 1987). These studies also showed that EDRF is sensitive to inactivation by oxygen or superoxide ( $O_2^-$ ). Thus redox agents which increase superoxide levels shorten the half-life whereas superoxide dismutase prolongs it (Moncada 1987; Furchgott, 1987). Haemoglobin added between the donor and detector also abolished the response, and this was thought to be due to binding of EDRF (Furchgott, 1987). EDRF was shown to possess similar characteristics (ie. all of the above) to nitric oxide, generated through nitrite acidification or injected as a gas (Furchgott, 1987). This led to the suggestion that EDRF and NO are one and the same (Furchgott, 1987). Comparative pharmacology, directly comparing NO with EDRF in tissue cascade systems supported this hypothesis, as the two were indistinguishable (Moncada, 1987; Furchgott, 1987). Confirmation came from measuring the release of NO from cultured endothelial cells using a chemiluminescent method (Moncada, 1987; Palmer, Ferridge & Moncada, 1987). The quantity of NO released in the effluent was found to be sufficient to account for the relaxant effects on the isolated strips. The discovery that EDRF is nitric oxide explains all the similarities to the nitrovasodilators and ensures that its postulated role as the endogenous nitrovasodilator is truly apt.

### Vascular smooth muscle contraction

In order to appreciate the intracellular mechanism of action of cGMP in mediating relaxation, something of the underlying basis of contraction in smooth muscle must be understood. In common with other types of muscle, contraction is initiated by an increase in the free cytosolic calcium concentration ( $[Ca]_i$ ). The rise in  $[Ca]_i$  is produced by an increase in Ca influx through the sarcolemma, together with release of Ca from intracellular stores. Calcium influx is controlled through both voltage operated (VOC) and receptor operated (ROC) channels in the sarcolemma. Depolarization of the membrane, through either an action potential, passive depolarisation or by drug induced increase in Na permeability, causes VOCs to open, thereby increasing Ca entry and causing contraction. On the other hand, hyperpolarisation, by a drug-induced increase in  $K^+$  permeability, closes VOCs and so reduces Ca influx and causes relaxation. Receptor operated channels, opened when a ligand binds to the receptor, increase Ca influx to a wide range of hormones and drugs (eg.  $\alpha$ -agonists, histamine, prostaglandins) without depolarisation of the membrane. It has also been suggested that operation of this type of channel is coupled to an increase in phosphoinositide turnover which mediates release of intracellular calcium through inositol trisphosphate formation (Downes & Mitchell, 1985; Huggins & England, 1985; Walker, Somlyo, Goldman, Somlyo & Trentham, 1987).

Intracellular release of calcium from sarcoplasmic reticulum (SR), mitochondria and membrane bound sites have been postulated to occur either through Ca-induced-Ca release

or through inositol trisphosphate metabolism (Huggins & England, 1985; Downes & Mitchell, 1985; Walker, Somlyo, Goldman, Somlyo & Trentham, 1987). The precise mechanism of excitation contraction coupling depends on the type of smooth muscle. In vascular smooth muscle, which does not show action potentials and has a sparse SR, contraction is thought to be regulated mainly by the interplay between calcium influx and efflux across the cell membrane.

A decrease in free  $[Ca]_i$  is produced by both Ca efflux and Ca sequestration into the SR. Calcium efflux is mediated through both a Ca-ATP'ase pump and a Na/Ca exchange mechanism in the sarcolemma, although the relative importance of each process is not known. Ca uptake into the SR is also mediated through a Ca-ATP'ase 'pump', although the relative contribution from the SR in decreasing  $[Ca]_i$  is unknown. The activity of both sarcolemmal and SR Ca-ATP'ases is stimulated by calmodulin (Wuytack, Schutter & Casteels, 1980; Rashatwar, Cornwell & Lincoln, 1987).

The regulatory action of  $[Ca]_i$  on the contractile proteins is mediated through the calcium binding protein calmodulin. This protein is very similar to troponin found in cardiac and skeletal muscle, and takes on a similar role. The Ca-calmodulin complex is an important regulator of many key enzymes, none more so than its activation of myosin light chain kinase (MLCK) which promotes contraction. The P-light chain of smooth muscle myosin must be phosphorylated before actin activation of myosin ATPase and contraction can occur. The extent of phosphorylation therefore determines how much acto-myosin interaction occurs and consequently how much

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tension is generated. Dephosphorylation of myosin P-light chains and hence inhibition of acto-myosin ATPase activity, is catalysed by phosphatases form I & II. The contractile state of the muscle depends on the relative activity of MLCK and the phosphatases. Increased  $[Ca]_i$  produces more Ca-calmodulin complex, which increases the activation of MLCK and so produces contraction. A decrease in  $[Ca]_i$  causes relaxation through a reduced activation of MLCK.

As has already been said, the actions of many hormones and drugs on smooth muscle contractility are mediated through alterations in the calcium influx. A different mechanism exists for the relaxant actions of  $\beta$ -adrenergic agonists which increase the intracellular concentration of cyclic adenosine 3-5-monophosphate (cAMP). The rise in cAMP activates cAMP-dependent protein kinase (cA-PK), an enzyme which catalyses the phosphorylation of a variety of proteins. One substrate for cA-PK is MLCK which is itself a phosphoprotein. Phosphorylation of MLCK by cA-PK results in a decrease in Ca-calmodulin stimulation of activity (Hathaway, Konicki & Coolican, 1985). Other substrates for cA-PK include membrane bound proteins, and these may be responsible for reducing Ca influx and increasing Ca extrusion and accumulation by the SR, although the exact molecular mechanisms have not yet been elucidated (Huggins & England, 1985). The major action in  $\beta$ -adrenergic relaxation is a reduced light chain phosphorylation via an attenuation of the Ca-calmodulin stimulation of MLCK activity.

The mechanism of relaxation by agents which increase cGMP is not so well understood. Initial suggestions that NP



mediated relaxation through hyperpolarisation were refuted when relaxation of rat tail artery by NP and 8-bromo-cGMP were shown not to affect the membrane potential (Cheung & MacKay, 1985). Identification of a cGMP dependent protein kinase (cG-PK) in smooth muscle suggested that protein phosphorylation was involved, similar to the mechanism of action of cAMP. Measurements of cG-PK activity in rat aortic strips showed that NP and ACH increased activity in a manner that correlated closely with the increased cGMP and relaxation (Fiscus, Rapoport & Murad, 1983). Further evidence came from a study which chromatographically isolated a spectrum of phosphoproteins from vascular smooth muscle preloaded with [ $^{32}$ P]ATP (Rapoport, Draznin & Murad, 1983). NP, ACH and 8-bromo-cGMP all elicited a similar pattern of protein phosphorylation which was associated with relaxation. Isoproterenol and dibutyryl-cAMP induced relaxation gave a different pattern of phosphorylation (Rapoport et al, 1982). Unlike cA-PK, purified cG-PK was shown to have no influence on the activity of MLCK, which suggested a different mechanism of action (Hathaway, Konicki & Coolican, 1985). Studies with Ca channel blockers (verapamil) and various external [Ca], led to the hypothesis that NP and 8-bromocGMP antagonised Ca influx and increased Ca sequestration and efflux (Lincoln, 1982). This was substantiated by recent measurements of  $^{45}$ Ca influx and efflux with tension showed that EDRF, NP & 8-bromo-cGMP caused relaxation by reducing both Ca influx and intracellular Ca release (Collins, Griffiths, Henderson & Lewis, 1986; Collins, Henderson, Lang & Lewis, 1988). In vitro studies have shown cG-PK activation of sarcolemmal Ca-ATPase

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in rat aortic cells (Rashatwar, Cornwell & Lincoln, 1987). A decrease in phosphatidylinositol hydrolysis has been shown to occur in rat aorta after application of NP, ACH, 8-bromo-cGMP and ANF (which activates particulate GC) (Rapoport, 1986). This process is linked to ROC opening and is postulated to be a mediator of intracellular Ca release.

Taken together, these reports suggest that phosphorylation by cG-PK reduces (i) Ca influx through ROC and VOC, (ii) attenuates intracellular Ca release, (iii) stimulates Ca accumulation by the SR, and (iv) increases extrusion of Ca via the sarcolemmal Ca-ATPase. These will all cause a marked reduction in  $[Ca]_i$  which will inactivate MLCK and so mediate relaxation. The exact molecular events underlying these observations have still to be elucidated.



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### Cardiac Muscle

The force of contraction of cardiac muscle, unlike skeletal muscle, is regulated by alterations in the force developed by each muscle cell. This can vary over a tenfold range without any change in muscle length or action potential height. The process of coupling excitation at the cell membrane to force generation at the contractile proteins is responsible for variations in contractile force induced by inotropic agents (ie. hormones, drugs and the ionic environment). As the current theories and controversies of excitation-contraction coupling in cardiac muscle have been reviewed by numerous authors, (Morad & Goldman, 1973; Ebashi, 1976; Fozzard, 1977; Fabiato & Fabiato, 1979; Chapman, 1979, 1983) only a short resume' of the major points of the theories are included in this introduction. The mechanisms by which various hormones and drugs affect this process will then be reviewed with particular emphasis on the role that cGMP plays in regulating cardiac contractility.

#### **Excitation contraction coupling**

In all muscle cells, contractile force is generated through interaction of the cross bridge head of the myosin (thick) filament with binding sites on the actin (thin) filament. In cardiac and skeletal muscles, the number of cross bridges formed is controlled by the tropomyosin-troponin regulatory protein complex associated with the actin filament. Troponin-I (Tn-I), in the relaxed state, masks the binding site on the actin molecule such that attachment of the myosin cross bridge head is prevented. This steric inhibition is removed when calcium binds to Troponin-C (Tn-C) which induces

a conformational change in the protein structure that moves Tn-I away from the active site. The number of cross bridges able to generate force is dependent on the amount of calcium bound to Tn-C.

The primary control of contraction is the free calcium concentration in the cytoplasm ( $[Ca]_i$ ) available to bind to Tn-C. This can be shown experimentally in 'skinned' muscle preparations where the sarcolemma is removed either chemically or by microdissection. The force produced by these preparations is directly related to the free calcium concentration ( $[Ca]_o$ ) of the bathing solution. Tension is developed at  $[Ca]_o$  greater than  $0.1\mu M$  and reaches a maximum of approx.  $100mN/mm^2$  at around  $20\mu M$  (Fabiato & Fabiato, 1975; 1979; 1982; Fabiato, 1981; McClellan & Winegrad, 1980; Morad & Goldman, 1973; Chapman 1983). Intracellular Ca-sensitive microelectrodes, have estimated the  $[Ca]_i$  of resting muscle as being  $0.2-0.3\mu M$  (Chapman, 1983). Fabiato (1981) compared the twitch tension of intact mammalian cells to the contracture produced after skinning, and showed that under optimal stimulation conditions (paired pulses), twitches can only produce 50-60% of the maximum contracture tension. Using the pCa-tension curve of the skinned preparations, this translated into a peak free  $[Ca]_i$  of  $4\mu M$  compared to the  $18\mu M$   $[Ca]_o$  required to fully activate the contractile proteins. Chapman (1983) after accounting for the Ca binding capacity of calmodulin, suggested that for Tn-C to be fully activated requires a rise in  $[Ca]_i$  of  $100\mu M$ . Comparing the tension generated by various preparations, he further suggested that the maximal rise in  $[Ca]_i$  was only  $25-50\mu M$  in mammalian

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fibres, but could reach 100uM in frog heart (Chapman, 1983). These results indicate that mammalian cardiac cells normally work well below their maximum force generating capacity so that increases in force can be induced by further increases in [Ca]<sub>i</sub>. Further evidence for this comes from experiments using the light-emitting Ca<sup>2+</sup> sensitive protein aequorin to directly measure the free [Ca]<sub>i</sub> in intact muscle cells. After the membrane is depolarised, the [Ca]<sub>i</sub> rises to a peak (130-220msec in frog or 20-25msec in mammalian muscles) which decays back to control levels at the peak of the tension response (Allen & Blinks, 1978). The reduction in free Ca is due to it rapidly binding to Tn-C (& calmodulin) to generate tension. Increases in twitch tension induced by increasing the extracellular calcium ([Ca]<sub>o</sub>), stimulation rate or applying isoprenaline are associated with larger transient increases in [Ca]<sub>i</sub> (Allen & Kurihara, 1980; Allen & Orchard, 1984; Morgan & Blinks, 1981).

The source of calcium responsible for the increase in [Ca]<sub>i</sub> is still a controversial issue, and shows considerable species differences. It is well established that the contractile strength of cardiac muscle, unlike skeletal muscle, is strongly dependent on the [Ca]<sub>o</sub>. The relatively large extracellular Ca concentration ([Ca]<sub>o</sub> = 1mM) coupled with a normal resting potential of -80mV, produces a large electrochemical gradient for Ca across the membrane ( $>10^6$ ) (Huggins & England, 1985). This has led to the suggestion that an influx of Ca through the sarcolemma is the major source of activator calcium. Calculations based on the Hill (1948) diffusion equations, estimate that the time for an influx of

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extracellular Ca to reach the required  $[Ca]_{i}$  50msec for frog but >1.3 sec for mammalian ventricular cells (Chapman, 1983; Fabiato & Fabiato, 1979) These times are compatible with the rate of tension generation and the aequorin signal for frog heart, but too slow for mammalian heart (Allen & Orchard, 1984). Influx of Ca from the extracellular fluid cannot directly contribute to tension development in the larger mammalian cells (10-15  $\mu$ m dia.), but can do so in the smaller diameter cardiac cells of the frog (2.5  $\mu$ m) (Morad & Cleeman, 1987).

The major route of trans-sarcolemmal influx of Ca is the inward Ca current during the plateau of the action potential (Morad & Goldman, 1973). The size of this current as measured by voltage clamp experiments is not adequate to raise the  $[Ca]_{i}$  by the required amount. Estimates of the rise in  $[Ca]_{i}$  by this route during a normal twitch, vary from 0.3-25  $\mu$ M in mammalian and 6-40  $\mu$ M in frog, significantly less in mammalian muscle than the 25-50  $\mu$ M required for a normal twitch (Chapman, 1983). Another route of Ca entry is through reversal of Na/Ca exchange across the sarcolemma. In the resting condition (membrane potential -80mV) this system operates to remove Ca from the cytoplasm, by allowing 3 Na<sup>+</sup> ions to flow down their electrochemical gradient in exchange for 1 Ca<sup>2+</sup> ion moving against its electrochemical gradient (Chapman, 1983; Caroni & Carafoli, 1983). However, during the action potential, depolarization of the membrane causes the electrogenic exchange to reverse direction and allow Ca to enter. Evidence for this comes from experiments in which the exchange is artificially reversed by depolarising

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the membrane for long periods (either by voltage clamp, raising  $[K^+]_o$ ) or by lowering  $[Na]_o$ . These procedures produce contractures in both mammalian and frog cardiac muscle which are associated with a rise in  $[Ca]_i$  and a measured outward current (Chapman, 1983; Chapman & Leoty, 1981; Morad & Cleeman, 1987). Long lasting depolarisations induce different kinds of contracture in amphibian and mammalian ventricular preparations. Frog ventricle maintains a steady high level of tension (tonic) which is related to the extent of depolarisation (Morad & Cleeman, 1987). However, frog atrial and mammalian preparations show an initial phasic component which rapidly declines to a secondary tonic phase (Chapman & Tunstall, 1981; Chapman, Coray & McGuigan, 1983; Flitney & Kennovin, 1984). The phasic component is associated with Ca release from the SR whereas the tonic phase is due to reversal of the Na/Ca exchange.

Low  $[Na]_o$  contractures in frog can increase the  $[Ca]_i$  enough to fully saturate Tn-C and generate maximum tension ( $100\text{mM/mm}^2$ ) (Chapman, 1983). Mammalian preparations by contrast produce small contractures in low  $[Na]_o$  which vary in relative size and only produce negligible rises in  $[Ca]_i$  ( $0.5\mu\text{M}$ ) (Chapman, 1983; Chapman, Coray, & McGuigan, 1983). This is thought to be due to the higher amount of SR present in mammalian tissue which quickly sequesters incoming Ca. The reversal of Na/Ca exchange can directly add to the activator Ca pool in frog cardiac cells, but indirectly in mammalian. It should be noted that most of the work on Na/Ca exchange has been done under unphysiological 'contracture' conditions and so its role in generating tension in the normal

twitch has yet to be fully elucidated.

The preceding arguments against an extracellular source of Ca for activation of tension in mammalian heart imply that an intracellular release site must be involved. In skeletal muscle, activator calcium comes entirely from the extensive sarcoplasmic reticulum (SR) system which releases it upon depolarisation through electrical couplings (triads) to the t-tubule system. Ultrastructural studies have revealed that SR is present in both frog and mammalian myocardium, but it is much less extensive in the former (0.5% fibre volume compared to 3.5% in mammalian; Morad & Cleeman, 1987). The SR takes up Ca through ATP dependent Ca pumps and has been shown to be able to accumulate up to 400uM per cell in mammalian but only 200 uM per cell in frog. The mechanism of its release is still a controversial issue. Mammalian myocardium does not possess triads, but instead there are close couplings with both the t-tubules and sarcolemma. Frog ventricle lacks a T-system and its sparse SR does not make direct connections with the sarcolemma.

Unlike skeletal muscle, depolarisation of the SR does not seem to be the mechanism that initiates Ca release. In partially skinned mammalian cardiac muscle cells, release of Ca from the SR can be induced by rapidly raising the bathing Ca concentration. This Ca-induced-Ca-release (CICR) can liberate enough Ca to generate 60% of maximum tension. Although this may be an underestimate, it is still more than enough to account for the release and uptake of Ca necessary for a normal twitch (Fabiato, 1981). From this evidence it has been suggested that in mammalian muscle, activator Ca is released



from the SR. The trigger for this is the rise in  $[Ca]_i$  due to influx of Ca during the action potential. The size of Ca-triggered contractions has been shown to correlate with the quantity of triggering Ca and the rapidity of its increase (Fabiato & Fabiato, 1975; Fabiato & Fabiato, 1979). Alterations in the size of the inward Ca current would therefore induce the SR to release varying amounts of activator Ca. The close proximity of the SR to the sarcolemma lends further evidence in support of this hypothesis.

Relaxation is associated with a decrease in  $[Ca]_i$  caused by uptake of Ca by the SR, or by extrusion of Ca across the membrane. Estimates of the rate and capacity of Ca accumulation by isolated SR show that it is more than adequate to relax a normal twitch in mammalian muscle. However, the mechanism whereby CICR is inactivated, to allow Ca accumulation, has still to be elucidated. The extrusion of Ca is thought to play little part in the relaxation mechanism of mammalian cardiac muscle.

CICR and rapid Ca accumulation by the SR cannot be demonstrated in frog ventricle (Fabiato & Fabiato, 1979). This lends further credence to the hypothesis that both activation and relaxation are due to Ca movements across the sarcolemma. The sparse SR in this tissue with its slow rate of uptake and release may serve as an intracellular Ca buffer. Relaxation is thought to occur by extrusion of Ca through the sarcolemmal Ca pump or Na/Ca exchange. Using isolated sarcolemmal vesicles, the maximal Ca flux carried by the sarcolemmal Ca pump was estimated to be 1/30-1/100 that of the Na/Ca exchange (Caroni & Carafoli, 1983).

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Another intracellular organelle that has been implicated in Ca homeostasis is the mitochondria. Mitochondria can accumulate and release large amounts of Ca through a Na/Ca exchange carrier in its membrane. It is thought however that they serve normally as a Ca buffer and are only involved in the release or accumulation of activator Ca when the  $[Na]_i$  is altered, for example, during Na withdrawal contractures or inhibition of the sodium pump (Chapman, 1983). An increase in  $[Ca]_i$  is thought to act as a second messenger inside mitochondria to stimulate oxidative metabolism and so promote the synthesis of ATP (McCormack & Denton, 1986). Increases in  $[Ca]_i$  therefore not only increase the energy requiring processes in cells (ie. contraction) but also stimulates production of the necessary fuel (ie. ATP) (Denton, McCormack, Hidgley, Rutter & Thomas, 1988).

In summary, the source of activator calcium is different in frog and mammalian cardiac muscle. In frog, extracellular calcium is utilised on a beat to beat basis via Ca influx through the slow inward calcium channel and reversal of Na/Ca exchange. In mammalian myocardium, these processes are only used to replenish the SR with calcium and the release of intracellular calcium activates contraction. These differences were highlighted recently in a comparative study of frog and mammalian excitation contraction coupling (Morad & Cleeman, 1987). After photolytic removal of nifedipine blockade of Ca channels, frog twitch tension fully redeveloped in the next beat, whereas 5-7 beats were required in mammalian ventricle (Morad & Cleeman, 1987). Sufficient Ca is transported from the extracellular space to fully activate



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tension in a single beat in frog whereas mammalian cells must first load up the SR before full recovery of tension is achieved.

As EC coupling in these tissues is fundamentally different, then inotropic agents or interventions would be expected to affect each one differently. A good example of this is post-extrasystolic potentiation which increases contractile strength in mammalian but not frog myocardium. Inotropic drugs or hormones will similarly affect each type of muscle differently depending on their intracellular mode of action.

### Role of cyclic 3'5'adenosine monophosphate (cAMP)

Following the discovery of its role in the regulation of liver metabolism, a similar 'second messenger' role for cAMP in heart muscle was proposed when its formation was shown to be increased by adrenaline (Murad, Chi, Rall & Sutherland, 1962). This was substantiated in the perfused working heart when adrenaline caused an increase in cAMP levels which preceded the positive inotropic response (Robison, Butcher, Oye, Morgan & Sutherland, 1965). It is now well established that  $\beta$ -adrenergic agents increase the force and rate of contraction and also the rate of relaxation in cardiac muscle through an increase in intracellular cAMP levels ([cAMP]<sub>i</sub>). Cyclic AMP is formed from ATP by the enzyme adenylate cyclase (AC) which is found in both SR and sarcolemmal membranes. Adrenergic agonist bound to  $\beta$ -receptors are now known to directly activate sarcolemmal AC through a membrane transducing protein (G-protein) (Neer & Clapham, 1988). The cAMP produced combines with the regulatory subunit of

cAMP-dependent-protein-kinase (cA-PK) which liberates the active catalytic subunit. The latter is responsible for phosphorylating a variety of regulatory enzymes (Corbin, Cobb, Beebe, Granner, Koch, Gettys, Blackmore, Francis & Wells, 1988). This introduction will concentrate on the substrates for cA-PK and how these are thought to influence various stages in the process of myocardial EC coupling.

#### Calcium influx.

Aequorin studies showed that the increased force of contraction induced by adrenergic agents correlated with an increase in the transient rise of  $[Ca]_i$  associated with contraction (Allen & Blinks, 1978; Morgan & Blinks, 1981; Allen & Orchard, 1984). The amplitude of the calcium transient is a measure of the amount of Ca liberated into the cytoplasm via the slow inward current ( $I_{Ca}$ ) of the action potential and by release from the SR. Indications of an increase in  $I_{Ca}$  was the prolongation of the action potential duration following adrenergic application that correlated well with the increase in twitch tension (Boyett, 1978; Reuter, 1979). Intracellular injection of cAMP also prolonged the duration, and raised the plateau of the action potential (Trautwein, Tangiguchi & Noma, 1982). Similarly, intracellular injection of cAMP into depolarised Purkinje cells induces slow action potentials which are mediated via  $I_{Ca}$  (Vogel & Sperelakis, 1981). Dibutryl cAMP was found to augment frog action potentials and  $I_{Ca}$  measured in voltage clamp conditions (Morad, Saunders & Weiss, 1981). Similar responses could be obtained following injection of the catalytic subunit of cA-PK supporting the hypothesis that phosphorylation of sarcolemmal Ca channel, or

an associated protein modifies its conductance (Bkaily & Sperelakis, 1984). Direct measurements of  $I_{Ca}$  using whole cell clamp techniques showed that  $\beta$ -adrenergic neurotransmitters increased the conductance of the channel without altering any of its other parameters (Reuter, 1983; Hartzell & Fischmeister, 1986; Morad et al, 1981). Detailed kinetic analysis of patch clamped or whole cell clamped myocardial preparations showed that  $\beta$ -agonists, cAMP and 8-bromo-cAMP increased the probability of Ca channel opening during depolarisation (Reuter, 1983).

Direct evidence that phosphorylation is involved in the  $\beta$ -adrenergic increase in  $I_{Ca}$  comes from the observation that cAMP dependent phosphorylation of a membrane protein in isolated sarcolemmal vesicles increases the calcium uptake induced by depolarisation (Rinaldi, Le Peuch & Demaille, 1981; Rinaldi, Capony & Demaille, 1982). A linear correlation was established between the cAMP dependent phosphorylation of a 23,000 dalton sarcolemmal protein and the voltage dependent Ca uptake by vesicles (Rinaldi et al, 1982). It is now thought that this protein is 'phospholamban' (meaning phosphate acceptor) which is known to regulate calcium transport in SR membranes (Huggins & England, 1985). Phosphorylation of phospholamban has been demonstrated in sarcolemmal vesicles rapidly isolated from  $^{32}P$  perfused intact hearts after isoprenalol treatment (Huggins & England, 1983). Phospholamban was the only phosphorylated protein and its phosphorylation by isoproterenol was rapid and preceded the positive inotropic response (Huggins & England, 1983). This is strong evidence that adrenergic agonists enhance contractility

through an increase in  $I_{Ca}$  via CAMP dependent phosphorylation of phospholamban.

#### SR release of calcium

The functional capacity of the SR has been investigated using mechanically skinned single myocardial cells which removes the sarcolemma but leaves the SR intact (Fabiato, 1981). Addition of CAMP increased the amplitude of the CICR contraction (by 78%), and decreased its duration (by 18%) with both a faster rate of tension development and relaxation (Fabiato & Fabiato, 1975; 1981). The tonic tension in low [EGTA] was also reduced by CAMP (Fabiato, 1975). In another skinned muscle preparation, caffeine induced tension transients were increased by CAMP, or the catalytic subunit of cA-PK, if present in the calcium loading solution (Su & Makencik, 1982). The increase in tension transient by CAMP was inhibited by the heat stable inhibitor of cA-PK (Su & Malencik, 1982). These studies support the hypothesis that CAMP stimulates accumulation of calcium by SR through phosphorylation of a membrane component. This can be recognised in intact myocardium by shortening of the augmented aequorin transient, with a reduction of the time to peak tension and a greatly accelerated decline of light intensity, following application of either noradrenaline, IBMX (PDE inhibitor) or dibutryl CAMP (Morgan & Blinks, 1981).

Calcium uptake into preparations of isolated cardiac SR vesicles is stimulated by CAMP, and this has been shown to be due to CAMP dependent phosphorylation of a membrane protein in SR of 22,00 dalton, called 'phospholamban' (Huggins & England, 1985). Phosphorylated phospholamban enhances the

activity of the Ca-transport ATPase, by increasing its affinity for calcium and the turnover rate of elementary steps in the ATPase reaction (Kranias & Solaro, 1982). The phosphorylation of phospholamban has been demonstrated in intact rabbit hearts perfused with  $^{32}\text{Pi}$ , from which highly purified SR vesicles were extracted at the peak of the inotropic response to isoproterenol (Kranias & Solaro, 1982). The incorporation of  $^{32}\text{Pi}$  into phospholamban was much greater than in control hearts and it paralleled the increase in contraction (Kranias & Solaro, 1982; Huggins & England, 1985).

The above evidence indicates that cAMP dependent phosphorylation of phospholamban increases the rate of calcium accumulation into the SR, which partly explains the increased rate of relaxation induced by  $\beta$ -agonists. A consequence of the increased calcium accumulation is that more is available for release in subsequent contractions thus mediating the increase in tension.

#### Phosphorylation of contractile proteins

Phosphorylation of cardiac TN-I was demonstrated in vivo in a number of species; the extent of phosphorylation increased after exposure to adrenaline and other  $\beta$ -agonists (England, 1980; Kranios & Solaro, 1982). Time course studies suggested a causal role of TN-I phosphorylation in the contractile regulation when the extent and rate of phosphorylation directly paralleled the increase in force of contraction of perfused whole rat hearts stimulated by adrenaline (England, 1975). It was proposed that TN-I phosphorylation might cause an increase in the affinity of

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TN-C for Ca, leading to a greater Ca-sensitivity of the contractile apparatus (England, 1975). Other positive inotropic agents (ie. cardiac glycosides or raised [Ca]<sub>o</sub>) failed to phosphorylate TN-I which supported the hypothesis that it was mediated through CA-PK (England, 1980). A study which measured TN-I phosphorylation in 'hyperpermeable' rat ventricular cells showed that CAMP did augment TN-I phosphorylation but that this was associated with a decrease in the Ca sensitivity of the contractile apparatus (Mope, McClellan & Winegrad, 1980). In intact myocardium, the relationship between the calculated [Ca]<sub>i</sub> (deduced from the aequorin signal) and tension, is lower for increases in contractility produced by adrenaline than those produced by raising [Ca]<sub>c</sub> or stimulation rate (Allen & Orchard, 1984). This was interpreted as a reduced Ca sensitivity of the contractile proteins. Phosphorylation of TN-I is now thought to be associated with the increased rate of relaxation induced by  $\beta$ -agonists and not the increase in tension.

In vitro measurements of acto-myosin ATPase activity showed that phosphorylation of TN-I caused a 5-fold increase in Ca concentration required for half maximal stimulation (Holrode, Howe & Solaro, 1979; England, 1980). The rate of dissociation of Ca<sup>2+</sup> from dephosphorylated TN-I is relatively slow ( $t_{1/2}$  = 50msec) and it is increased by phosphorylation ( $t_{1/2}$  = 30msec) (Huggins & England, 1985). This ensures that the rate of Ca<sup>2+</sup> dissociation from the myofibrils is still faster than the increased rate of uptake by the SR such that the overall relaxation rate is increased (Huggins & England,



1985). The increase in the maximal velocity of relaxation over that of contraction after  $\beta$ -agonist or dibutryl cAMP treatment, was shown to correlate with the cAMP-PK activity and increased [cAMP]<sub>i</sub> in whole rat hearts (Argel, Vittone, Chiappe, Cingolani & Grassi, 1980).

The myosin P-light chain is also phosphorylated in heart muscle (Jeacocke & England, 1980; England, 1980; Opie, 1982). The precise role of phosphorylation of this contractile protein has not been established. Adrenaline was shown not to alter the level of P-light chain phosphorylation in rat heart (Jeacocke & England, 1980). A small increase in P-light chain phosphorylation was reported for rabbit heart, but this reached a maximum after the peak of the inotropic response (Westwood & Perry, 1981). There are reports however that light chain phosphorylation increases actomyosin ATPase activity (Resink, Gevers, Noakes & Opie, 1981). Opie (1982) reviewed the evidence for and against a role for P-light chains and suggested that the negative effects of TN-I phosphorylation would mask any positive effects from myosin LC phosphorylation, so that it is probably not an important regulator of contractility.

In summary,  $\beta$ -agonist induced increase in force of contraction is mediated through a cAMP-dependent increase in  $\text{Ca}^{2+}$  influx, which is later taken up by the SR. Increased rate of relaxation is mediated through a decrease in Ca affinity of the contractile proteins and an increase in the rate of Ca sequestration by the SR. This scheme would increase the contractile response in both mammalian and amphibian myocardium although the lack of an effective SR in the latter

could present problems in accounting for the increased rate of relaxation (Morad, Saunders & Weiss, 1981). An increase in Ca efflux may however be involved in the adrenergic response, as sarcolemmal ATPase activity has been shown to be stimulated by cA-PK phosphorylation of phospholamban (Caroni & Carafoli, 1981; Lamers, Stinis & Jonge, 1981). Although activation by cAMP can increase Ca efflux through the Ca-pump, its low activity as compared to Na/Ca exchange would suggest that even after phosphorylation its contribution would be small. In this last regard, phosphorylation of Na/Ca exchange has been demonstrated to increase its exchange activity in vitro (Caroni & Carafoli, 1983). This is mediated through a Ca-calmodulin dependent kinase present in the sarcolemma, and so would only be indirectly affected by  $\beta$ -adrenergic stimulation through increased [Ca]<sub>i</sub>.

#### Role of calmodulin

Calmodulin is a ubiquitous and multifunctional Ca dependent regulatory protein, capable of activating a wide variety of key enzymes (Cohen, 1982). Upon an increase in cytosolic [Ca]<sub>i</sub> from pCa8 to pCa5, calmodulin binds Ca and forms an active Ca-calmodulin-enzyme ternary complex, which results in increased activity of that enzyme (Walsh, Le Peuch, Vallet, Cavadore & Demaille, 1980). The functions of calmodulin therefore depend on the particular substrate enzymes present, and this differs in individual tissues. In cardiac muscle, Ca-calmodulin is thought to activate a membrane bound protein kinase (present in both SR and sarcolemma), myosin light chain kinase, sarcolemmal Ca-ATPase and a cyclic nucleotide phosphodiesterase (PDE I) which hydrolyses both



cAMP and CGMP. (Walsh et al, 1980; Cohen, 1982). As phosphorylation of the myosin light chain does not play a major role in regulating contractility, the main actions of calmodulin are associated with Ca fluxes and the regulation of cyclic nucleotide levels.

Most of the calmodulin effects on regulating Ca movements are similar to those of cAMP, in fact they augment one another at two important sites. The regulation of  $I_{Ca}$  is one such site. A calmodulin-dependent protein kinase in the sarcolemma mediates this effect by phosphorylating phospholamban at a different site to that of cA-PK (Cohen, 1982). The importance of calmodulin in regulating  $I_{Ca}$  was shown by the ability of the calmodulin inhibitor calmidazolium to completely abolish slow action potentials in cultured heart cells (Bkaily & Sperelakis, 1986). Full recovery of these action potentials required both calmodulin and the catalytic subunit of cA-PK (Bkaily & Sperelakis, 1986). Similarly, the activity of CaATPases is also dependent on the dual phosphorylation of phospholamban (Walsh et al, 1980; Wuytack, DeSchulter & Casteels, 1980; Lamers, Stinis & Dejonge, 1981; Caroni & Carafoli, 1981). The extraction of calmodulin from cardiac sarcolemmal vesicles or the addition of trifluoperizine (TFP) results in a reduction of the Ca affinity of CaATPase (Caroni & Carafoli, 1981). The stimulatory effects of cAMP or calmodulin on Ca-ATPase are independent of each other (Lamers et al, 1981). Phosphorylation of SR phospholamban by the Ca-calmodulin dependent protein kinase alone results in activation of the Ca pump, whereas that mediated by cA-PK alone does not. However,

cA-PK amplifies the activation induced by calmodulin (Walsh et al, 1980). It is clear from such studies that cAMP-dependent regulation of Ca influx, Ca sequestration and Ca efflux are all strongly affected by calmodulin. The action of calmodulin in vivo has been assessed by studying the effects of calmodulin blockers on the  $\beta$ -adrenergic responses in rat papillary muscles (Aass, Skomedal & Osnes, 1983). Calmodulin inhibition attenuated the increase in relaxation but augmented the positive inotropic response (Aass et al, 1983). It was postulated that these effects are due to reduced rates of Ca sequestration and Ca efflux.

Calmodulin also plays a role in the regulation of cyclic nucleotide metabolism as it stimulates the activity of a cyclic nucleotide phosphodiesterase (PDE). Of the three PDE's present in cardiac muscle, calmodulin stimulates PDE I. This enzyme hydrolyses both cAMP and cGMP, although the substrate affinity varies with species (Weishaar, Burrows, Kobylarz, Quade & Evans, 1986). Guinea-pig ventricular PDE I hydrolyses both nucleotides to similar degrees, whereas rat ventricular PDE I preferentially hydrolyses cGMP (Weishaar et al, 1986; Terasaki & Appleman, 1975). This has important implications when considering the calmodulin stimulation of activity as it will cause an increase in [cAMP] over [cGMP] in the rat but not in the guinea-pig. Inhibition of calmodulin in the frog ventricle elevated cGMP levels by a greater extent than cAMP levels and produced a decrease in twitch tension (Flitney, Moshiri, Robertson & Singh, 1981). This suggests a similar form of PDE I in frog as in rat. In mammalian ventricle, Ca-calmodulin stimulation of PDE activity does not

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appear to have the same importance, as calmodulin inhibitors (TFP or chlorpromazine) did not increase cAMP levels in perfused rat heart, with or without stimulation by isoprenaline (Werth, Hathaway & Watanabe, 1982).

In summary, the actions of calmodulin in mammalian muscle augment those of cAMP, namely: increasing Ca influx, Ca sequestration and Ca efflux. However, in amphibian ventricle, calmodulin may play a major role in controlling the levels of cyclic nucleotides.

### Role of cGMP in cardiac muscle.

The first indication of a role for cGMP in regulating myocardial contractile activity was the finding that the decrease in twitch tension after perfusing rat hearts with acetylcholine (Ach) correlated with increased intracellular cGMP levels (George, Polson, O'toole & Goldberg, 1970). Further studies showed that this response was also characterised by a decrease in cAMP levels (George, Wilkerson & Kadowitz, 1972). Lipid soluble cGMP analogues (8-bromo-cGMP) mimic the decrease in tension produced by Ach, whereas cAMP analogues increase tension, similar to adrenergic stimulation (Nawrath, 1976). Similar studies led to the hypothesis that cGMP mediates the inhibitory actions of the parasympathetic neurotransmitters analogous to the proposed role of cAMP in mediating sympathetic responses. Goldberg (1975) proposed the 'ying-yang' hypothesis that suggests cAMP and cGMP have opposing actions in intracellular regulation (Goldberg, Haddox, Nicol, Glass, Sanford, Kochl & Estensen, 1975).

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Subsequent studies have however criticised this hypothesis as they have shown a lack of correlation between intracellular cGMP and contractility (Diamond, TenEick & Trapani, 1977; Linden & Brooker, 1979). Consequently, Drummond and Severson in a 1979 review have stated "no definite role of this cyclic nucleotide in cardiac function is yet apparent". In recent years, some progress has been made in identifying the intracellular actions of cGMP, but its precise role in the regulation of myocardial contractility remains obscure.

Results from the frog ventricle provide the best evidence for a 'ying-yang' hypothesis of cyclic nucleotide regulation of myocardial contractility. Since 1977, Flitney and Singh have provided an increasing body of evidence in support of the idea that both cAMP and cGMP are involved in regulating ventricular contractility. The major evidence for this is that all inotropic agents or procedures, both positive and negative, alter the ratio of endogenous [cAMP]:[cGMP] in a manner that correlates precisely with the change in twitch tension. Positive inotropic agents (adrenaline, isoprenaline, ATP, UTP) or procedures (stretch), increase the ratio whereas negative inotropic agents (Ach, adenosine, NP) or procedures (hypodynamia), decrease the ratio (Flitney, Lamb & Singh, 1977a,b;1979; Singh, Flitney & Lamb, 1978; Flitney & Singh, 1978;1979; 1980a,b,c,d,e; Flitney, Moshiri & Singh, 1980a,b; Singh & Flitney, 1980a,b;1981). The magnitude of the responses are all paralleled by quantitatively equivalent changes in the ratio. The time course of these effects also correlates precisely, even in the complicated triphasic tension responses to ATP, the change in tension is quantitatively equivalent to

the change in the nucleotide ratio throughout (Flitney & Singh, 1980(c)). Evidence that this is a causal relationship is that predetermined changes in the ratio can be induced by applying lipid soluble analogues of either nucleotide. The magnitude of the decrease in tension produced by 8-bromo-cGMP, or the tension increase produced by dibutryl cAMP, are each paralleled by quantitatively similar changes in the ratio of the total nucleotides (ie. endogenous and exogenously applied nucleotides). The increase in cGMP produced by Ach or 8-bromo-cGMP is accompanied by a marked decrease in the cAMP level, whereas elevated cAMP levels by isoprenaline or dibutryl cAMP slightly raised cGMP levels (Singh et al, 1978; Flitney & Singh, 1981). Treatment with the phosphodiesterase (PDE) inhibitor theophylline, attenuated the effects of 8-bromo-cGMP and Ach on endogenous cAMP levels (Flitney & Singh, 1981). This was taken as evidence that cGMP reduces cAMP levels by stimulating cAMP PDE activity (Flitney & Singh, 1981). Identification of a cAMP PDE in rat heart and other tissues, whose activity could be stimulated 6-10 fold by cGMP supported this hypothesis (Terasaki & Appleman, 1975). If such an enzyme is present in frog ventricle, then it could account for the conformity of the cyclic nucleotide ratio. The possibility that endogenous cGMP regulates cAMP hydrolysis is suggested as one of the underlying mechanisms in the cyclic nucleotide control of contractility. Other direct functions of cGMP in regulating contractility must also exist however, or the contractile response would correlate more closely with changes in cAMP alone.

In mammalian cardiac muscle the role of cGMP is less

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clear. Early studies suggested a role for both nucleotides in the regulation of mammalian contractility, but few authors measured intracellular cAMP and cGMP concentrations simultaneously (see Diamond & Severson, 1979). Recently, Rodger & Shahid (1984) measured both in rabbit papillary muscles and tried to correlate them to the changes in contractility produced by various inotropic agents. Isoprenaline induced dose dependent increases in tension and both [cAMP] and [cGMP] (Rodger & Shahid, 1981). Direct stimulation of adenylate cyclase with forskolin increased cAMP, without affecting cGMP levels. In both cases tension correlated closely with intracellular [cAMP] and was not related to the ratio of [cAMP]:[cGMP]. Direct stimulation of guanylate cyclase with NP elevated cGMP levels and slightly decreased cAMP, but failed to produce any inotropic effect. In contrast to results with frog ventricle, these results would suggest that cGMP plays no part in regulating contractility in mammalian ventricle (Rodger & Shahid, 1984). The direct role of total [cAMP]i in mediating contractility has also been questioned. Although there are good correlations between [cAMP]i and tension, a similar increase in force of contraction was produced by isoprenaline and forskolin, despite cAMP levels being 8 times higher with the latter agent (Rodger & Shahid, 1984). This indicates that intracellular cAMP may be a determinant of increased contractility, but the relationship between the two is not one of simple proportionality.

There are numerous reports showing that in mammalian atrial muscle, intracellular cGMP is not associated with the



decrease in tension produced by various agents. Diamond, TenEick & Trapani (1977), showed that low doses of Ach decreased the twitch in cat atria without affecting cGMP or cAMP levels. In the same preparation, NP induced large increases in cGMP with no change in cAMP, and produced a slight (11%) increase in twitch tension (Diamond et al, 1977). In isolated guinea-pig atria, carbachol (3 $\mu$ M) reduced twitch tension by over 80% without affecting cGMP levels (Linden & Brooker, 1979). Similarly, rat atrial twitch is almost abolished by high doses of methacholine which produce no increase in cGMP levels (Linden & Brooker, 1979).

A comparative study on the action of muscarinic agonists (carbachol), NP and 8-bromo-cGMP on canine atria and ventricle showed that these agents increase cGMP in both tissues, but only decreased the twitch in atrial preparations (Endoh & Yamashita, 1981). The size of the twitch depression did not correlate with the increase in cGMP, as carbachol almost abolished the twitch (91% depression) but only elevated cGMP 1.5 times (30-50fmol/mg), whereas NP increased cGMP 40 fold (43-1800fmol/mg) but only reduced tension by 22% (Endoh & Yamashita, 1981). The positive inotropic action of various agents (PE, isoprenaline, glucagon, papverine & caffeine) were antagonised by carbachol, but not by NP or 8-bromo-cGMP (Endoh & Yamashita, 1981). A similar study in guinea pig showed that adenosine and related analogues decreased the force of contraction and reduced the action potential duration in atria but not in ventricle, although the cAMP or cGMP did not change in either tissue (Bruckner, Fenner, Meyer, Nobis, Schmitz & Scholz, 1985). In rabbit, carbachol increased cGMP, without



affecting cAMP, in both atria and ventricular preparations, but only reduced the twitch in atrial tissue (MacCleod & Diamond, 1986). After treatment with forskolin, carbachol was able to induce negative inotropic responses in both preparations. This latter effect of carbachol was blocked in ventricular muscle by the cGMP reducing agent LY83583 but not in atrial muscle (MacCleod & Diamond, 1986).

All the above evidence would suggest that cGMP generated through muscarinic stimulation (or some direct action not involving cGMP) is involved in the regulation of normal contractility of atrial muscle, but not ventricular muscle. The 'antiadrenergic' action of muscarinic agonists is mediated through cGMP in ventricular muscle but not in atrial. Other agents which induce increases in cGMP not associated with muscarinic stimulation (ie. NP or 8-bromo-cGMP) appear not to affect contractility in either type of cardiac muscle. It would seem therefore that there are different intracellular pools of cGMP in mammalian myocardium, and the one associated with muscarinic stimulation is more important in regulating contractility.

The large discrepancy in the effects of intracellular cGMP between amphibian and mammalian preparations could be the result of two circumstances:- (i) The processes involved in excitation contraction coupling in frog are more susceptible to modulation by cyclic nucleotides than in mammalian myocardium. The greater dependence on trans-sarcolemmal movement of 'activator' calcium in frog would appear to be more sensitive to cGMP regulation (see later). (ii) The more highly ordered structure of mammalian myocardium, with greater

amounts of intracellular membranes and organelles, together with the larger size of the cell (16-40 fold larger volume) would tend to compartmentalise the cell to a much greater extent than in frog. It has already been suggested that different pools of cGMP are responsible for the differences between mammalian atrial and ventricular muscle, it is logical to propose that a similar (or even greater) difference could exist between mammalian and amphibian preparations.

In order to compare these suggestions, an insight into the intracellular actions of cGMP would be useful.

#### Intracellular actions of cGMP

**Cyclic GMP dependent protein kinase** If cGMP regulates myocardial contractility in an analogous way to that of cAMP, then this requires the presence of a cGMP dependent protein kinase (cG-PK) in heart muscle. Such an enzyme was shown to exist in substantial amounts in mammalian heart (Lincoln, Hall, Park & Corbin, 1976). It was postulated that as cA-PK activity correlates well with positive  $\beta$ -adrenergic actions, then cG-PK should correlate with negative inotropic actions of muscarinic agonists (Lincoln & Keely, 1980). It was reported that whereas both NP and Ach increased the [cGMP] of rat heart (8- and 3-fold respectively), only Ach raised the activity of cG-PK and decreased the force of contraction (Lincoln & Keely, 1981). This suggests that only the cGMP pool produced by Ach is coupled to protein kinase activity and that this activity is related to the decrease in the twitch (Lincoln & Keely, 1981). The lack of coupling of cGMP produced by NP to cG-PK activity is thought to be responsible for its lack of

inotropic efficacy, which suggests that some pools of cGMP are not compartmentalised with the enzyme (Lincoln & Keely, 1981). It would appear that guanylate cyclase (GC) coupled to muscarinic receptors is more available to cG-PK, and is functionally different, than the GC that is activated by NP. This might suggest that GC activated by muscarinic receptors is membrane bound (particulate GC) as it has been shown in vitro that particulate GC is unresponsive to NP stimulation (Waldman & Murad, 1987). The total cGMP level of a tissue is not as physiologically relevant as that intracellular pool which is accessible to cG-PK. Similarly, physiologically relevant changes in pools of cyclic nucleotides can occur upon hormonal stimulation without significant changes in the total cyclic nucleotide levels. This effect is not unique to cGMP as different cAMP pools are now thought to be associated with different agonist effects. Isoproterenol (10nM) and PGE<sub>1</sub> (30uM) increase [cAMP] to approximately the same concentration in perfused rabbit heart, but only isoproterenol increased force of contraction. Furthermore, both agonists produced different 'spectra' of activities in a variety of cA-PK substrates (Brunton, Hayes & Mayer, 1981). This was thought to involve different pools of cAMP and differing discrete pools of cA-PK (not just particulate and soluble) (Brunton et al, 1981). The discrepancy in total [cAMP] compared to tension discussed earlier also suggests compartmentation of different cAMP pools (Rodger & Shahid, 1984). Brunton et al (1981) offered a plausible explanation for these results, namely that "the precise spatial and temporal orientation of hormone receptors, adenylate cyclase, cAMP, protein kinases, and

protein kinase substrates into functional compartments contribute to the specificity of cardiac contractility and metabolism by cAMP (Brunton, Hayes & Mayer, 1981).

Another problem with the proposed role of cG-PK in cardiac muscle regulation is the lack of demonstrated substrates for the enzyme. In contrast, smooth muscle cG-PK has been shown to phosphorylate numerous proteins, some of which have been identified as key regulators of contractility (ie. MLCK). In cardiac muscle however only one endogenous substrate protein of molecular mass 70,000, has been found in the cytosol of rat heart (Wrenn & Kuo, 1981). Further purification of this protein is required before its function can be determined. Other cytosolic substrates are conspicuous by their absence, which may infer that most of the substrates for this enzyme are particulate, although there is no evidence for this at present.

#### cGMP dependent phosphodiesterase (PDE II)

There are three main types of PDE in cardiac muscle. PDE I is similar to that found in smooth muscle and platelets, and its activity is calmodulin dependent. PDE I isolated from guinea-pig ventricle hydrolyses cAMP and cGMP equally, unlike that isolated from rat ventricle which preferentially hydrolyses cGMP (Weishaar, Burrows, Kobylarz, Quade & Evans, 1986; Terasaki & Appleman, 1975). Cardiac PDE II has high  $K_m$  and  $V_{max}$ , it is not stimulated by calmodulin and has no substrate specificity (Weishaar et al, 1986). It is this enzyme which is thought to act as a cGMP activated cAMP PDE (Terasaki & Appleman, 1975). The enzyme shows positive cooperativity for its two substrates (cAMP & cGMP). However,

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because the substrate affinity for cGMP is 2-3 fold below its reported level in myocardium and due to the higher cAMP concentration, increases in low concentrations of cGMP activate cAMP hydrolysis (Terasaki & Appleman, 1975). This enzyme is suggested to be responsible for cGMP regulation of cAMP levels and the basis behind the 'ying-yang' hypothesis (Terasaki & Appleman, 1977). The third PDE III only hydrolyses cAMP and it is not stimulated by calmodulin. Recent reports suggest that there are different subclasses of this enzyme which are associated with different domains of the cell (Manganiello, 1987). The subclasses are differentiated by their susceptibility to inhibition by various 'cardiotonic' drugs. For example, imazodon and cilostamide inhibit one particular subtype, whereas RO 20-1724 and rolipram inhibit another (Weishaar et al, 1987).

The imazodan-sensitive subtype of PDE III is also inhibited by cGMP (Weishaar et al, 1987). Imazodan produces a marked positive inotropic action in canine ventricular preparations, but only exerts a modest positive inotropic action in guinea-pig. This is taken as evidence for functional compartmentalisation of the enzyme as this subtype is particulate in dog, but present in the cytosol of the guinea-pig (Weishaar et al, 1987).

Cyclic GMP could therefore regulate cAMP hydrolysis through two completely different PDE's, as it would enhance hydrolysis by PDE II, but attenuate hydrolysis by imazodan sensitive PDE III. With the discovery of different intracellular locations of PDE associated with different functional pools of nucleotides, only the cGMP present in the

same 'compartment' would affect cAMP hydrolysis and this would have a variable effect depending on the associated function of that compartment. The use of specific PDE inhibitors may be the key to unravelling the complex network of separate functional pools of nucleotides.

#### Effect of cGMP on slow inward calcium current

The reduction in action potential duration seen with muscarinic agonists led to the proposal that cGMP decreases the slow inward calcium current ( $I_{Ca}$ ) (Ikemoto & Goto, 1977). This is supported by experiments showing that intracellular injection of cGMP, or extracellular application of dibutryl cGMP, both shortened the action potential duration (APD) in isolated ventricular (guinea pig and rabbit) cells (Trautwein, Taniguchi & Noma, 1982). The lack of any effect of cGMP on Ach induced hyperpolarisation supports the hypothesis that cGMP is involved in the regulation of voltage operated Ca channels and does not affect the classical muscarinic increase in potassium channel conductance (Trautwein et al, 1982). Intracellular injection of cGMP, or perfusion with 8-bromo-cGMP, abolished slow action potentials, which implicated direct inhibition of the voltage dependent Ca channel (Wahler & Sperelakis, 1985). This was in direct opposition to the augmentation of  $I_{Ca}$  and slow action potentials seen with intracellular injection of cAMP (Vogel & Sperelakis, 1980). Contradictory results were obtained by measuring  $I_{Ca}$  in voltage clamped frog trabeculae, in which the [cAMP]<sub>i</sub> or [cGMP]<sub>i</sub> was rapidly increased by photolysis of a membrane permeable 'caged' derivative (Nargeot, Nerbonne, Engels & Lester, 1983). Intracellular



concentration jumps of cAMP rapidly (<150msec) increased the amplitude of  $I_{Ca}$  but similar increases in cGMP failed to have any effect (Nargeot et al, 1983). Measurements of  $I_{Ca}$  in frog ventricle, using the whole cell clamp technique agreed with this result, in that intracellular perfusion of cGMP had no effect on  $I_{Ca}$  (Hartzell & Fischmeister, 1986). However, if  $I_{Ca}$  is previously elevated by  $\beta$ -agonists or intracellular cAMP, then cGMP markedly reduces the amplitude of the current (Hartzell, 1986). Similarly, Ach also had no effect on basal  $I_{Ca}$ , but it greatly reduced  $I_{Ca}$  previously elevated by isoprenaline (Fischmeister & Hartzell, 1986).  $I_{Ca}$  was also increased if cAMP was introduced into the patch pipette, but this increase could not be reduced by Ach (Fischmeister et al, 1986). This was taken as evidence that muscarinic agonists antagonise the  $\beta$ -adrenergic induced elevation of  $I_{Ca}$  by reducing intracellular cAMP levels. As cGMP could not reduce the  $I_{Ca}$  elevated by the non-hydrolysable 8-bromo-cAMP, nor can it reduce cAMP elevated  $I_{Ca}$  in the presence of a phosphodiesterase inhibitor (MIX), it was proposed that cGMP stimulation of cAMP-PDE, caused a reduction in cAMP, which consequently reduced  $I_{Ca}$  (Fischmeister & Hartzell, 1987). This hypothesis agrees with that discussed earlier for the opposing roles of cAMP and cGMP in regulating contractility in this tissue (Flitney & Singh, 1981). Since Ca influx is the major source of activator calcium in frog heart, then this mechanism would be an important determinant of contractile tension. However, in mammalian myocardium with its functional compartmentalisation of both nucleotides and activator calcium (SR), this mechanism would be less important.



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### Other postulated actions of cGMP

Ach has been shown to markedly inhibit the isoproterenol-induced phosphorylation of phospholamban in mixed SR and sarcolemmal vesicles from guinea pig ventricle (Watanabe, Lindeman & Fleming, 1984). The decreased phosphorylation was shown to be associated with a reduced Ca-ATPase activity of the vesicles and was thought to due to decreased cAMP levels (Watanabe et al, 1984). This was postulated to be due to a reduction in adenylate cyclase activity, but it could equally well result from a cGMP mediated increase in cAMP hydrolysis. This mechanism would explain some of the 'antiadrenergic' actions of muscarinic agents and cGMP.

In hyperpermeable rat ventricular preparations, cGMP is postulated to activate a phosphatase which decreases the phosphorylation of TN-I (Mope, McClellan & Winegrad, 1978). This serves to increase the Ca sensitivity of the contractile apparatus and opposes the action of cAMP which phosphorylates TN-I. The physiological significance of this is unclear as an increase in Ca sensitivity would augment contractile tension. It may however be responsible for antagonising the enhanced relaxation seen with  $\beta$ -agonists and so could mediate part of the 'antiadrenergic' action.

### Summary

In summary, the physiological significance of cGMP in regulating myocardial contractility is much less clear than for cAMP. However, in frog ventricle it would appear that cGMP is as important as cAMP in regulating contractility. Evidence

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is growing that this is mediated through a cGMP dependent cAMP PDE (PDE II) which suggests that cGMP only modifies the concentration of cAMP and hence has an indirect effect. While this may account for the inverse relationship demonstrated between the two nucleotides, it cannot account for the close empirical relationship between the ratio of cAMP:cGMP and tension. This suggests that cGMP must have other more direct actions in regulating contractility, as tension would otherwise correlate more closely to cAMP alone. The reliance of frog EC coupling on extracellular influx of calcium, and the demonstration that this is closely regulated by both nucleotides, is further evidence for a regulatory role for cGMP. A single pool of both nucleotides (as NP and 8-bromo-cGMP mediate effects) responsible for regulating  $I_{Ca}$  and tension suggests that the system is more rudimentary than mammalian heart.

The role of cGMP in mammalian myocardium is still obscure. The in vitro evidence would suggest that all the chemical regulatory systems are geared towards cGMP having an opposing role to cAMP, but demonstrations of these effects in situ has proven difficult. This may be because of two important issues:

1. Functional compartmentalisation of both nucleotides hinders investigation as any nucleotide measurement may not be related to function. The possibility that in each separate 'pool' there exists a complex sequence of reactions and interactions to elicit a particular response means that each system must be treated as an entity in itself. Progress can therefore only be made when specific systems can be identified with

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particular functions without contamination from other systems (ie. the GC stimulated by Ach and not NP, and vice versa).

2. Muscarinic agonists mediate effects that are independent of cGMP. Muscarinic receptors are known to directly interact with key effectors without involving production of cGMP. They are thought to do this through specific guanine nucleotide binding proteins (G-proteins) which are present in the sarcolemma and act as 'go-betweens' coupling receptors to appropriate membrane bound effectors (Houslay, 1987; Neers & Clapham, 1988). Three types of G-proteins are thought to interact with the muscarinic receptor:  $G_i$  - which inhibits adenylate cyclase activity in the membrane;  $G_k$  - increases potassium conductance;  $G_o$  - inhibits voltage dependent Ca channels (Houslay, 1987). Presumably there must be another muscarinic G-protein responsible for stimulating guanylate cyclase, but that has not been identified yet.

Adenylate cyclase inhibition by muscarinic interaction with  $G_i$  has been demonstrated in whole hearts and cardiac sarcolemmal vesicles (Watanabe, Lindeman & Fleming, 1984). The muscarinic antagonism of  $\beta$ -agonists increase in adenylate cyclase activity in isolated vesicles was dependent on the presence of GTP, which is known to be an important cofactor in the coupling of G-proteins to receptors (Neers & Clapham, 1988). The direct regulation of potassium channels by muscarinic agonists may have important implications in their reported ability to decrease  $I_{Ca}$  and action potential duration without altering cGMP levels.  $G_o$  has been shown to inhibit voltage sensitive Ca channels in nervous tissue, its presence in heart muscle could explain the direct effects of Ach on

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cAMP mediated  $I_{Ca}$  in whole cell clamp preparations (Fischmeister & Hartzell, 1987). Taken together, the direct actions of muscarinic receptors via G-proteins obviously lessens the role of cGMP as the second messenger of the parasympathetic system", and could explain some 'dissociation' results of cGMP and response.

## CHAPTER 2.

### Material and methods

## 1. Experiments on frog ventricular trabeculae

### i. Animals & Dissection

Experiments were performed using isolated ventricular trabeculae from the common frog *Rana Temporaria*. Prior to use, animals were kept at four degrees centigrade in shallow tanks to avoid the need for feeding. Each frog was first stunned by a blow to the head before being killed by decapitation and pithing. The heart was rapidly excised and washed in Ringer's solution (composition: NaCl 115mM, KCl 2.5mM,  $\text{NaH}_2\text{PO}_4$  2.15mM,  $\text{Na}_2\text{HPO}_4$  0.85mM,  $\text{CaCl}_2$  1mM, NaPyruvate 5mM, pH 7.2) at room temperature (18-22°C). After the atria and ventricle were separated, the ventricle was sliced vertically into two to expose the inner sponge-like surface. Free-running trabeculae of between 20-100 microns in diameter and 2-3 mm in length were then dissected out, with clumps of tissue at either end to facilitate mounting. Ventricular trabeculae differed from those in the atria in that they were smaller and generally not free along their length, so that it was harder to obtain single preparations. Bundles of two or three trabeculae were therefore sometimes used but only if they all had the same rest length and contracted synchronously.

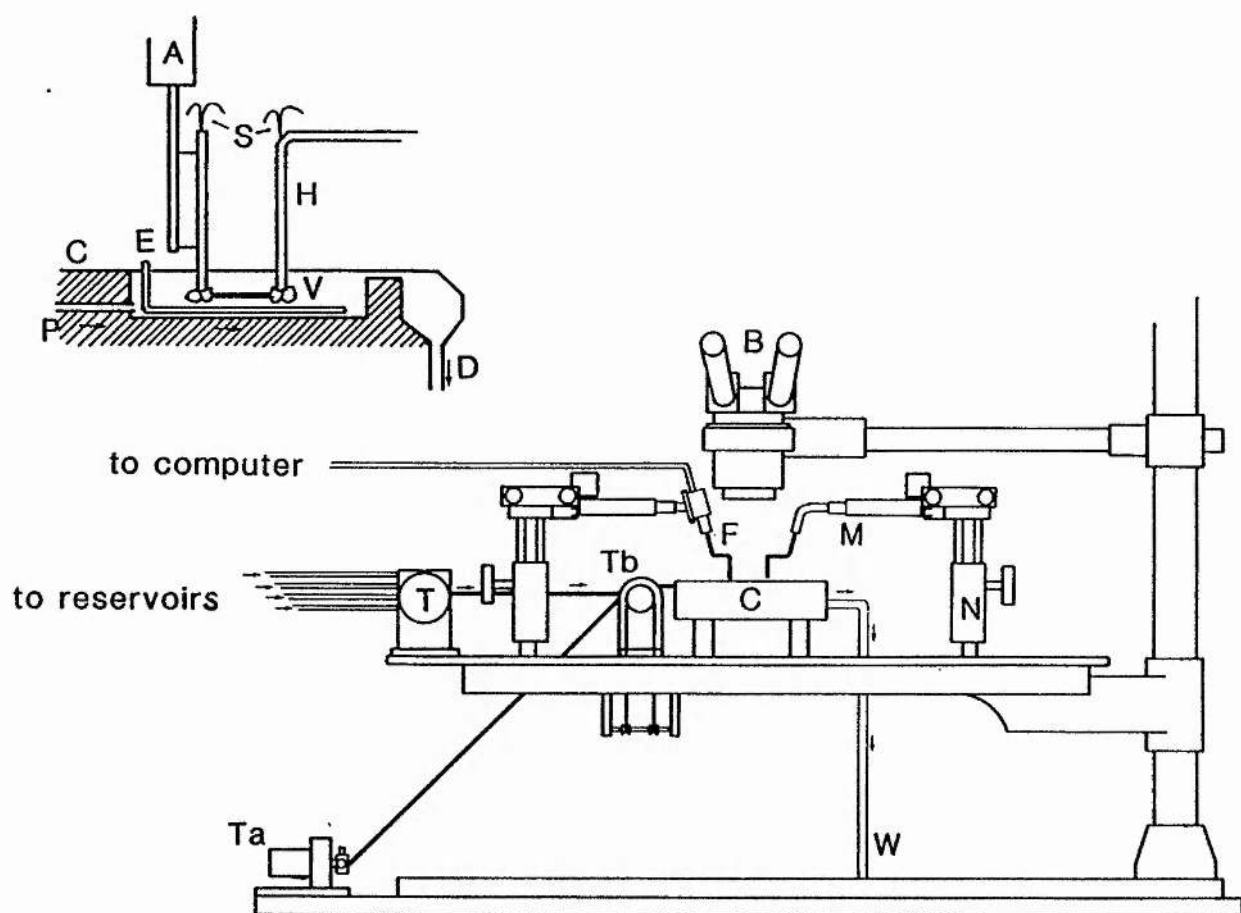
### ii. Apparatus

The isolated trabeculum was mounted between two metal supports using a snaring technique modified from Chapman & Tunstall (1971). The supports were made from syringe needles (21 gauge) down which was glued thin stainless steel tubing (29 gauge) which held the tungsten wire snares (13 micron diam. wire; Goodfellow Metals)(inset fig.2:1). One of the

Figure 2.1

- C - Perfusion chamber (inset showing cross section)
- E - Platinum wire stimulating electrodes
- S - Tungsten wire snares
- H - Syringe needle metal supports
- A - Force transducer (Akers)
- V - Trabeculum
- D- Drain
- M - Microdrives
- N - Micromanipulators
- F - Force transducer (Pixie)
- T - Six way tap
- B - Binocular microscope
- T<sub>a</sub> & T<sub>b</sub> - Computer controlled motorised taps
- W - Waste





## Chapter 2

supports was attached to a force transducer while the other was fixed to a rigid support (see fig.2:1). Two types of transducer, based on either a Pixie or Akers strain gauge, were used. The Akers was slightly more sensitive although more fragile than the Pixie but both used the same electronics and holder system and had similar characteristics. The overall compliance of either apparatus was 0.01 mm/mN, equivalent to a 0.25% change in length for a trabeculum 2mm long producing a force of 0.5mN (50mg). The output of the transducer was amplified (3dB point, 15Hz) and fed simultaneously to a Gould digital oscilloscope, a JJ pen recorder and a Unilab computer interface. The latter allowed the signal to be digitised, displayed and later analysed by a BBC microcomputer. The data collection program automatically measured and stored the peak twitch tension produced by the muscle and had an option for selecting and storing complete twitches.

The required perfusate was selected by means of a six way tap connected to six reservoirs through individual constant flow devices. Since the chamber was gravity fed, these kept the flow constant by maintaining the same height of solution above the level of the chamber (see fig.2:2). This was important as it prevented 'offset' artefacts when changing solutions. The normal flow rate was 10ml/min. which due to the small size of the bath completely changed the bathing solution every 0.5 sec..

### iii. Protocol

The isolated trabeculum was transferred to the perfusion chamber in a small bath, and then mounted by snaring the clumps of tissue at either end as shown in the inset of

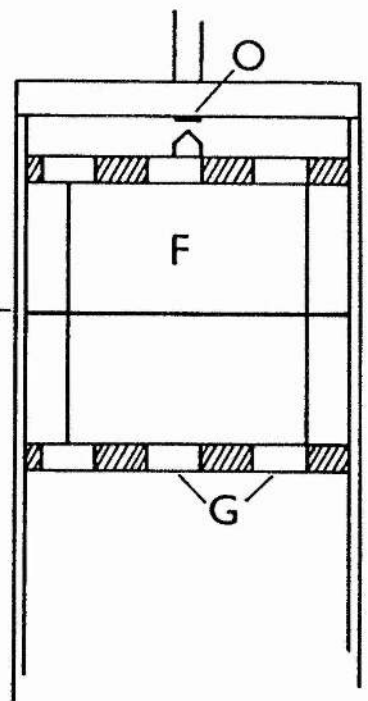
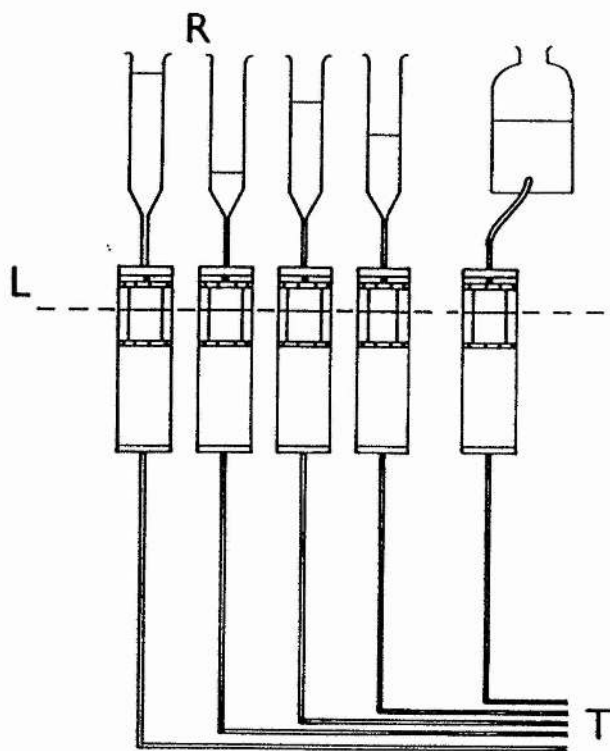
Figure 2.2

R - Reservoirs for each different solution. Note the different levels

L - Constant level of solution in each float chamber ensuring the same head of pressure.

T - To the six way tap

The close up of the float chamber in the right hand panel shows how the constant level of perfusate is maintained. Solution from the reservoir flows into the chamber through the O-ring (O), and passes over the float (F) through cut groves in it's surround (G). As the efflux of fluid from the chamber is slower than the influx from the reservoir, the chamber fills with solution. The chamber continues to fill, thus raising the float, until the float needle is pushed into the O-ring which impedes the inflow of solution. If the level of solution then falls, due to perfusing the bath, the descent of the float needle allows some more fluid in from the reservoir until the original level is attained. The float therefore oscillates, switching the inflow from the reservoir on and off and thus maintaining the level of solution constant.



## Chapter 2

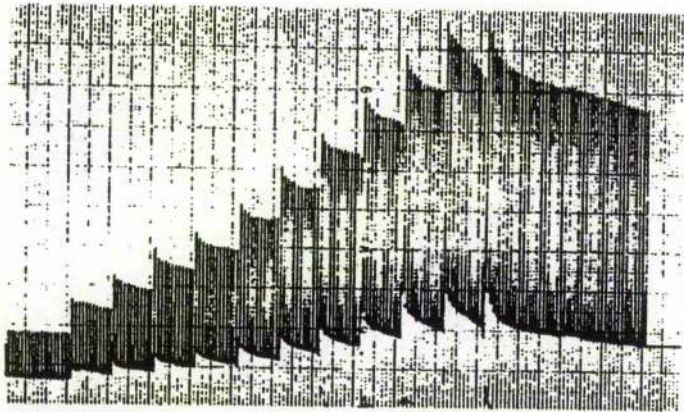
fig.2:1. Using the micromanipulators the mounted muscle was manoeuvred quickly into the perfusion chamber, taking care to ensure that it was never stretched or allowed to dry out. Once in position, the muscle was stretched to its normal rest length (ie. stretched until all the kinks just straighten out). Isometric twitch contractions were elicited by field stimulation through platinum wire electrodes fixed to the inner walls of the chamber. The stimulus voltage was increased from zero until the muscle started to twitch. The final voltage was set at double this threshold value. The normal stimulation parameters were a 12-25 V pulse of 1-2 msec duration every 5 sec (0.2 Hz).

After an initial stabilization period of 15min, the length of the trabeculum was stretched by 0.1 mm at 1 minute intervals, until the optimal twitch tension was obtained. A typical chart recording of this procedure is shown in fig.2:3 (top panel), which also shows the same event as recorded by the computer (bottom two panels). As the computer only records the twitch height, it effectively removes the change in resting tension, leaving only the change in active tension. The shape of any individual twitch could be stored by the computer as illustrated in the bottom right panel of fig.2:3. The length-tension relationship for 27 preparations determined in this way is shown in figure 2:4. Each muscle was left for a further 30min to settle down before commencing the experiment. The addition of pyruvate to the ringer solution prevented the onset of hypodynamia (Clarke, 1913) so that the constant twitch height remained constant throughout an experiment (Ventura-Clapier & Vassort, 1980a,b).

## Chapter 2

### Figure 2:3

The top panel is an original chart recording showing the increase in isometric twitch tension when muscle length is increased stepwise, in increments of 0.1mm every minute. The increase in muscle length is illustrated in the middle panel, while the bottom panel shows the same trace as recorded by the computer. The change in resting tension shown in the chart recording is ignored by the computer such that a clearer measure of the active tension is obtained. The bottom right hand panel is superimposed computer recordings of the shape of twitch contractions selected at the end of every second length change.

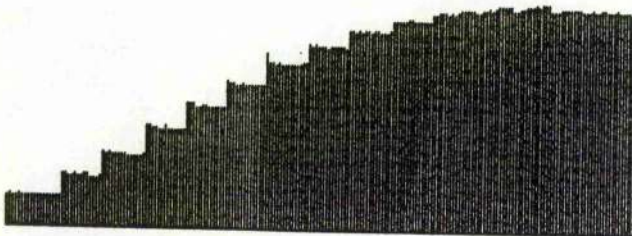


| 0.1mN

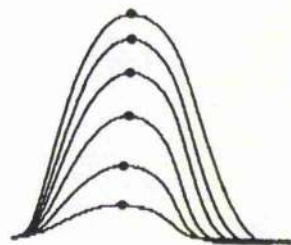
1min



| 0.5mm



0.5sec



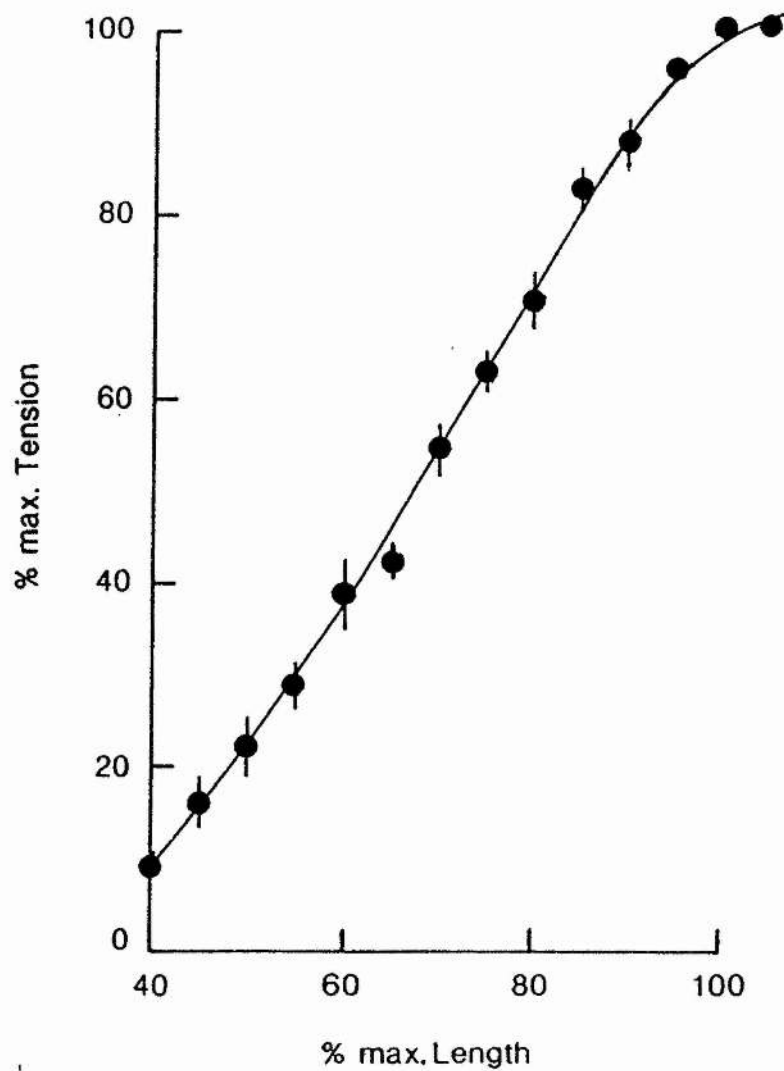
| 0.1mN



## Chapter 2

### Figure 2:4

This graph shows the mean (+ S.E.) normalised length tension relationship for 27 trabeculae.



### CHAPTER 3.

Preliminary observations of the effects  
illumination has on the response of frog  
ventricle to nitroprusside.

Introduction

Previous studies from this laboratory have shown that changes in the relative concentrations of intracellular cyclic 3'5'-nucleotides are associated with changes in the strength of the twitch contraction of isolated frog ventricular muscle (Flitney, Lamb & Singh, 1977a,b; 1978a,b; 1979). The ratio of the concentrations of adenosine 3'5'cyclic monophosphate (cAMP) to guanosine 3'5'cyclic monophosphate (cGMP) correlates closely with changes in the size of the twitch. This was shown to hold true for numerous inotropic agents, both positive and negative, and also for the gradual decline in twitch tension during the development of the hypodynamic state (Flitney, Lamb & Singh, 1977a,b; 1978; Flitney & Singh, 1978; 1979; 1980a,b,c,d,e). Even the time course of the tension response to these agents was found to follow closely the time course of the change in the cAMP/cGMP ratio. These observations suggest that both nucleotides are involved in mediating the change in twitch tension and that they function antagonistically, cAMP augmenting twitch contractions and cGMP attenuating them. Evidence for a causal relationship, was obtained when the ratio was changed in a controlled manner by applying lipid soluble analogues of either cAMP or cGMP. Both of these induced the appropriate change in the twitch tension (Flitney & Singh, 1980a; 1981). On the basis of these results, Flitney and his co-workers concluded that both cAMP and cGMP are involved in regulating the inotropic status of the frog ventricle.

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Flitney et al thought they would test their hypothesis with an agent that increased the cGMP concentration. Sodium Nitroprusside (NP) had been shown to directly stimulate guanylate cyclase activity both in vitro and in vivo (Murad, Arnold, Mittal & Braughler, 1979). They found that SNP reduced the size of the twitch and increased the concentrations of both nucleotides, cGMP being raised more than cAMP such that the ratio was reduced (Flitney, Moshiri & Singh, 1980a). The rate and size of this effect was however much greater on exposing the preparation to the same solution of SNP for a second time. NP was known to be photosensitive, and so the experiments were conducted under darkened room lighting with much of the apparatus covered with aluminium foil. The results now to be described are from experiments which were undertaken to confirm these observations using a better preparation, with improved apparatus and under more carefully controlled lighting conditions. Flitney, Moshiri & Singh (1980) had shown that NP is an extremely potent negative inotropic agent, which can exert an effect at  $1\text{nM}$ . The results now to be presented however, show that it is completely inotropically inert even at concentrations  $10^7$  fold greater than this, if sufficient care is taken to prevent its photolysis.

Methods

NP was prepared by dissolving crystalline sodium nitroprusside in low sodium Ringer (112mM) so that a  $10^{-2}$ M stock solution with a normal sodium content (118mM) was obtained. This was later diluted to the appropriate concentration with normal Ringer's solution. Both procedures were carried out in darkness with the only means of illumination being dull red light from a small hand torch. Solutions were then either protected from light or exposed to light using one of the following two methods:-

i. The NP solution was exposed to light before use. This was done in a controlled way, by constantly stirring a 1 litre conical flask of SNP solution (at the desired concentration) in front of two 60W desk lamps placed 20cm away for a period of 1 hour.

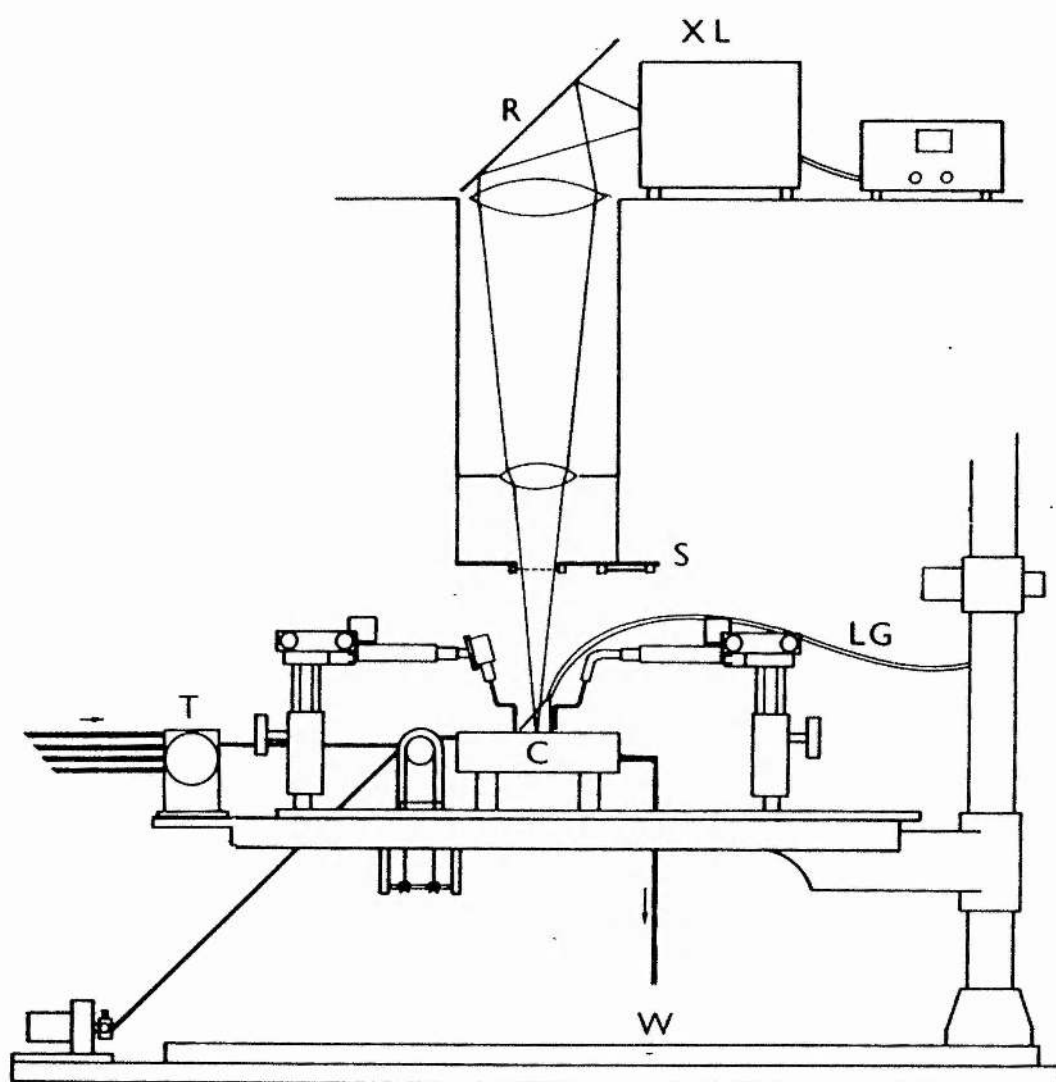
or

ii. The solution was prepared in darkness and exposed as it flowed through the muscle chamber. Initially this was done using the fibre optic light guide (see fig. 3.1) which was the normal source of illumination. When greater light intensities were required, a 150W xenon arc lamp was used which was focused onto the chamber through a slide mount (see fig. 3.1). This enabled the relative intensity and colour of the light to be altered by inserting neutral density and/or spectral filters (Ilford gelatin: Nos.600-608) in the path of the incident beam. Both types of experiment were carried out in a darkroom with a red photographic safelight as the only means of illumination.

Figure 3:1

The perfusate, selected by means of the six-way tap (T), flows in the through the muscle chamber (C) and down to waste (W). It can be illuminated in the muscle chamber by light from either the fibre optic light guide (LG) or the xenon arc lamp (XL). Early experiments used light from the fibre optic which is normally only used for setting up the preparation. The source of light for this is a 150 W tungsten filament halogen bulb (not shown) which gives a poorer illumination than the xenon lamp. Light from the xenon arc lamp was reflected off a mirror (R) and focused by two lenses before passing through a slide mount (S). The intensity and wavelength of the incident light can be altered by putting neutral density or spectral filters in the path of the beam. The whole apparatus was enclosed in a solid aluminium faraday cage that prevented any extraneous light entering or leaving the site of exposure.





Results

i. Effects of unexposed nitroprusside.

Sodium nitroprusside solutions that had been carefully protected from exposure to light had no effect on the twitch. This is illustrated in figure 3:2, which shows computer recordings from two experiments. In both cases the peak twitch tension is unaffected when the bathing solution is changed from normal Ringer's solution (R) to one containing  $10^{-2}M$  sodium nitroprusside solution (R+NP).

ii. Effects of nitroprusside pre-exposed to 'white' light.

Panel A in figure 3:2 show recordings from an experiment in which a solution of  $10^{-2}M$  NP was pre-exposed to light before it was applied to the muscle (R+NP<sup>\*</sup>). Perfusing a preparation with this solution caused the strength of the twitch to slowly decrease, reaching a new steady state level of around 42% of the initial control twitch after 15 minutes.

Re-perfusing with protected NP, allowed the twitch to recover to its pre-treated control level. This emphasizes the inert nature of unexposed NP as it can washout the effects of the physiologically-active pre-exposed NP.

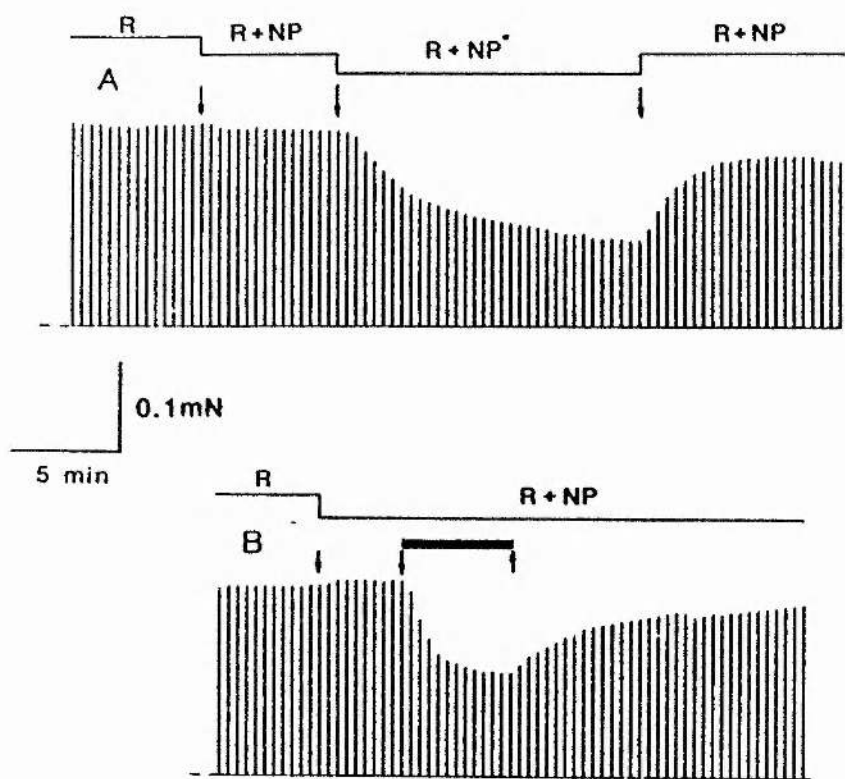
This procedure was repeated for concentrations of NP ranging from  $10^{-5}$  to  $10^{-2}M$ . The log dose response curve (see fig.3:5) obtained is sigmoidal in shape with an  $ED_{50}$  value of  $5 \times 10^{-4} M$  NP. The maximum twitch depression was  $57\% \pm 2.0\%$  (S.E.:  $n=12$ ).

figure 3:2

The two panels of this diagram show computerised recordings of the peak twitch height with time. Each vertical bar indicates the peak tension of every fifth twitch according to the scale bar on the left. The time interval between each bar is 25 seconds. The top trace in each case marks the perfusate changes, a downward deflection of the bar along with an arrow showing when a changeover occurs.

Panel A shows the effect of changing the perfusate from normal Ringer (R) to Ringer containing  $10^{-2}$ M NP that had been totally protected from light (R+NP). There is no change in the strength of the twitch. Changing the perfusate to Ringer containing  $10^{-2}$ M NP that had been pre-exposed to light (R+NP\*), the twitch tension gradually decreases to reach a new steady level after 15 minutes. Changing the perfusate back into protected NP solution (R+NP) recovers the twitch back to normal.

Panel B illustrates responses from an experiment in which the NP was exposed to light while it was flowing through the muscle chamber. Ringer containing  $10^{-2}$ M NP that had been totally protected from light does not affect the twitch as before. Illuminating this solution in the muscle chamber with the light guide (thick black bar), caused the twitch tension to quickly decrease to a new steady level. Once the light is switched off, the twitch tension recovers back to control levels.



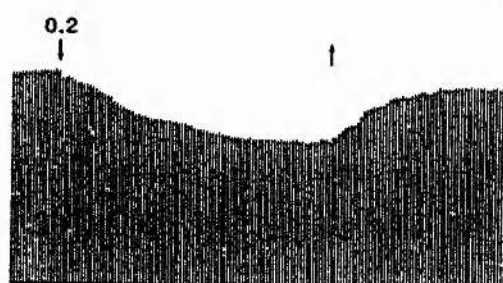
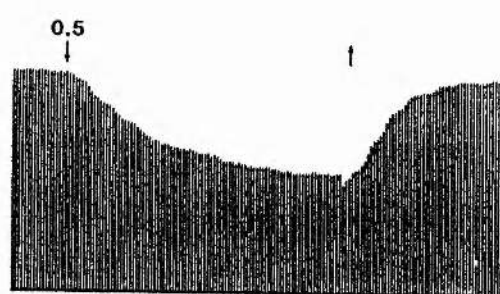
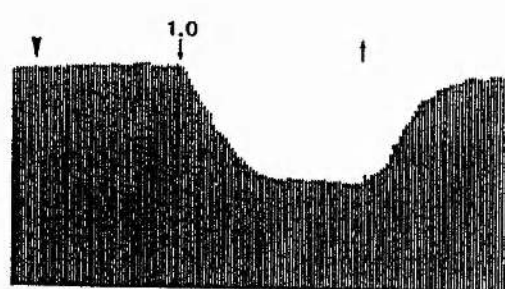
iii. Effects of directly exposing nitroprusside to white light in the perfusion chamber.

Panel B in figure 3:2 shows the effect of exposing NP solutions to white light in situ, ie. while it is in the bath and in close proximity to the muscle. The twitch was again unaffected by changing the perfusate from normal Ringer (R) to Ringer containing  $10^{-2}$ M protected NP (R+NP). Light from a tungsten halogen lamp was then directed onto the preparation through a light guide. Twitch tension immediately started to decline at the onset of illumination and continued to do so throughout the duration of the exposure (black bar; fig.3:2) until a new steady level was reached. The new twitch tension was 52% of the original control twitch and was reached 5min. after the onset of illumination. On switching the light off (end of black bar), twitch tension immediately began to recover, even though the muscle remained in unexposed nitroprusside solution. Complete recovery normally took approximately 6 mins.

This experiment was repeated using a number of different intensities of light to illuminate the muscle chamber. Neutral density filters were placed in front of a 150W Xenon arc lamp (see methods), so as to vary the amount of light reaching the preparation. An example of this type of experiment is shown in figure 3:3. Four traces are shown here to illustrate the responses obtained by illuminating the same NP solution ( $10^{-2}$  M) in the perfusion chamber, with different intensities of white light. The top left hand panel shows the changeover from normal Ringer's solution to one containing protected NP (thick

Figure 3:3

This computer record shows four sequential traces from the same experiment, each illustrating the effect of illuminating  $10^{-2}M$  protected NP to varying light intensities. The large downward arrow indicates the changeover of perfusate from normal ringer to ringer containing  $10^{-2}M$  protected NP, after which the perfusate was not changed again throughout the remainder of the experiment. The small downward arrow indicates the start of illumination of the perfusate in the muscle chamber, the number immediately above giving the relative intensity. The upper left hand response was with the full intensity of the xenon lamp and so has a relative intensity of 1.0. Lower relative intensities of 0.5, 0.2 & 0.1 were produced by placing 2-, 5- & 10- fold attenuating neutral density filters in front of the incident beam. Both the rate and extent of the depression of the twitch were reduced by reducing the intensity of illumination as is shown in the other three panels. The small upward arrow indicates the end of illumination after which the twitch tension always recovers, even though the perfusate is still NP solution.



1min.  
0.05 mN



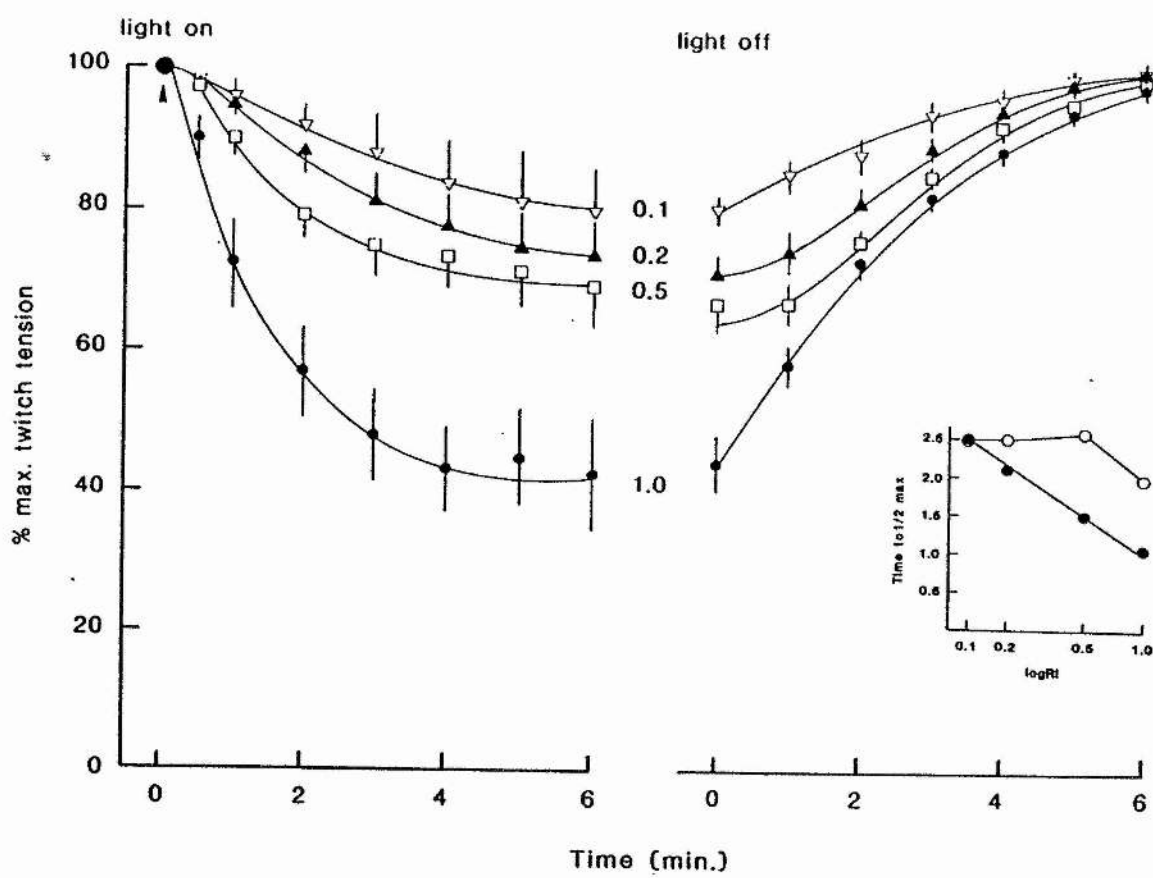
### Chapter 3

arrow). Again, protected NP does not alter the twitch tension, until such time as it is exposed to light, some four minutes later (downward arrow). In this panel there was no filter to attenuate the light (relative intensity = 1) which reduced the twitch tension to 47% of its control value. On switching the light off (upward arrow) the twitch fully recovered whilst the preparation was still being perfused with protected NP solution. The remaining panels show three, sequential exposures of the perfusate to decreasing levels of illumination. Reduction of the incident intensity, decreased both the rate and extent of the twitch depression. This is more clearly seen in the averaged responses shown in figure 3:4. This shows the time course of the decrease in twitch tension after the perfusate was exposed to light of varying intensities. At maximum illumination (relative intensity 1.0), the twitch tension rapidly decreases, with a half time of 1.1 min, to 42(+ 8)% of its pre-illumination value. Lower intensities (relative intensities of 0.5, 0.2 & 0.1) reduce the twitch to an extent which is related to the amount of illumination (69%, 75% & 81% of control respectively). The rate of depression of the twitch is also related to the intensity of illumination as the higher intensities produce the faster decrease in tension. The inset shows the plot of the log of the intensity against the time for half maximum response. The linear relationship indicates that there is an exponential increase in the rate of depression of the twitch when the intensity of illumination is increased. When the illumination ceases (right hand portion of fig 3:4), the twitch recovers back to control levels while still in unexposed NP solution.

Figure 3:4

Plotted here is the average (+ S.E.) twitch tension of 6-12 preparations, sampled each minute up to 6 minutes during and after illumination of a  $10^{-2}$ M NP perfusate. The response to four different intensities of illumination have been superimposed to show that the rate and extent of depression of the twitch is dependent on the intensity of illumination. The relative light intensity of each averaged response is indicated beside the respective trace.

The inset shows a plot of the log of the relative intensity against the time to half maximal response for both the depression (solid circles) and recovery (open circles). The linear relationship suggests that the rate of depression varies exponentially with the intensity of illumination. The rate of recovery however seems to be more independent of the intensity of illumination.



### Chapter 3

These data show that the rate of recovery is approximately the same, except that for the maximal illumination intensity. This suggests that the recovery process may be a washout phenomenon, governed by the rate of diffusion of the physiologically active agent out of the muscle. The reason for the faster rate of recovery at the greater depression is not known.

Similar responses were obtained using various concentrations of SNP in the solution. The resulting log dose response curves from these are shown in figure 3:6. The top curve is the log dose response curve for a relative intensity of 1.0. It has the normal sigmoidal shape with an  $ED_{50}$  value of approx.  $5 \times 10^{-4}M$ . This curve is comparable to that for the results obtained by pre-exposing NP solutions (white dots) described earlier. The log dose response curves for the lower intensities (0.5 & 0.2) show reduced responses at each NP concentration but the  $ED_{50}$  values are similar for all three curves.

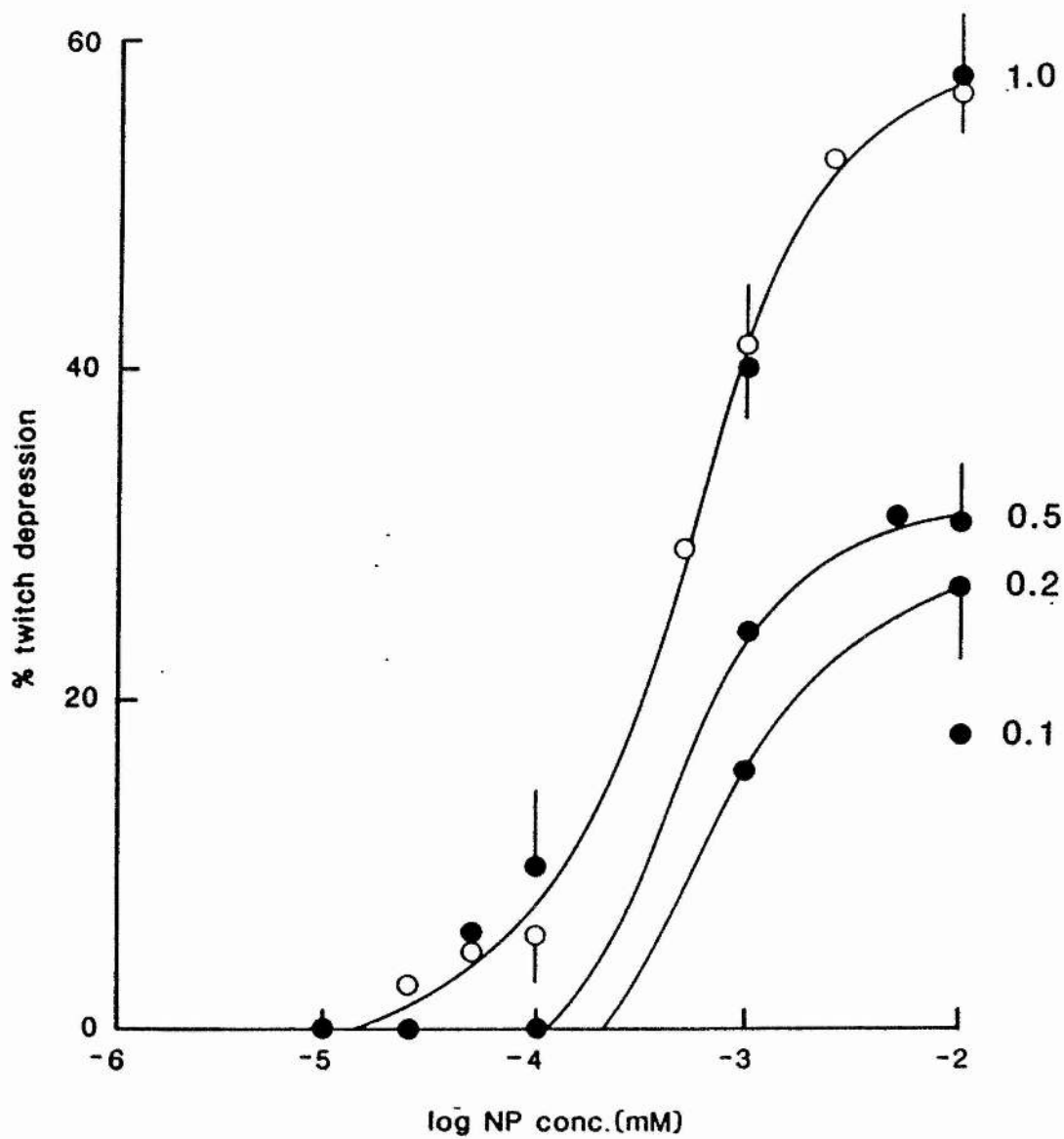
#### iv. Effect of directly exposing NP to light of different wavelengths.

The above results clearly indicate that NP must be exposed to light before it can exert any effect on the twitch. An experiment was therefore conducted to see whether this was influenced by the wavelength of the illuminating light. A  $10^{-2}M$  NP solution was exposed to light of different spectral content by passing the beam through different coloured filters (see methods). A suitable combination of neutral density filters was used to ensure all the colours were of the same

### Chapter 3

#### Figure 3:5

The log dose response curves to SNP that had been pre-exposed (open circles) and directly exposed (closed circles) to various light intensities are shown here. Pre-exposure for 1hr or direct exposure to the full intensity of the xenon lamp causes the same maximal depression of the twitch (58%) and has the same  $ED_{50}$  value of  $5 \times 10^{-4} M$ . Lower intensities have a lower maximal effect but the same  $ED_{50}$  value.



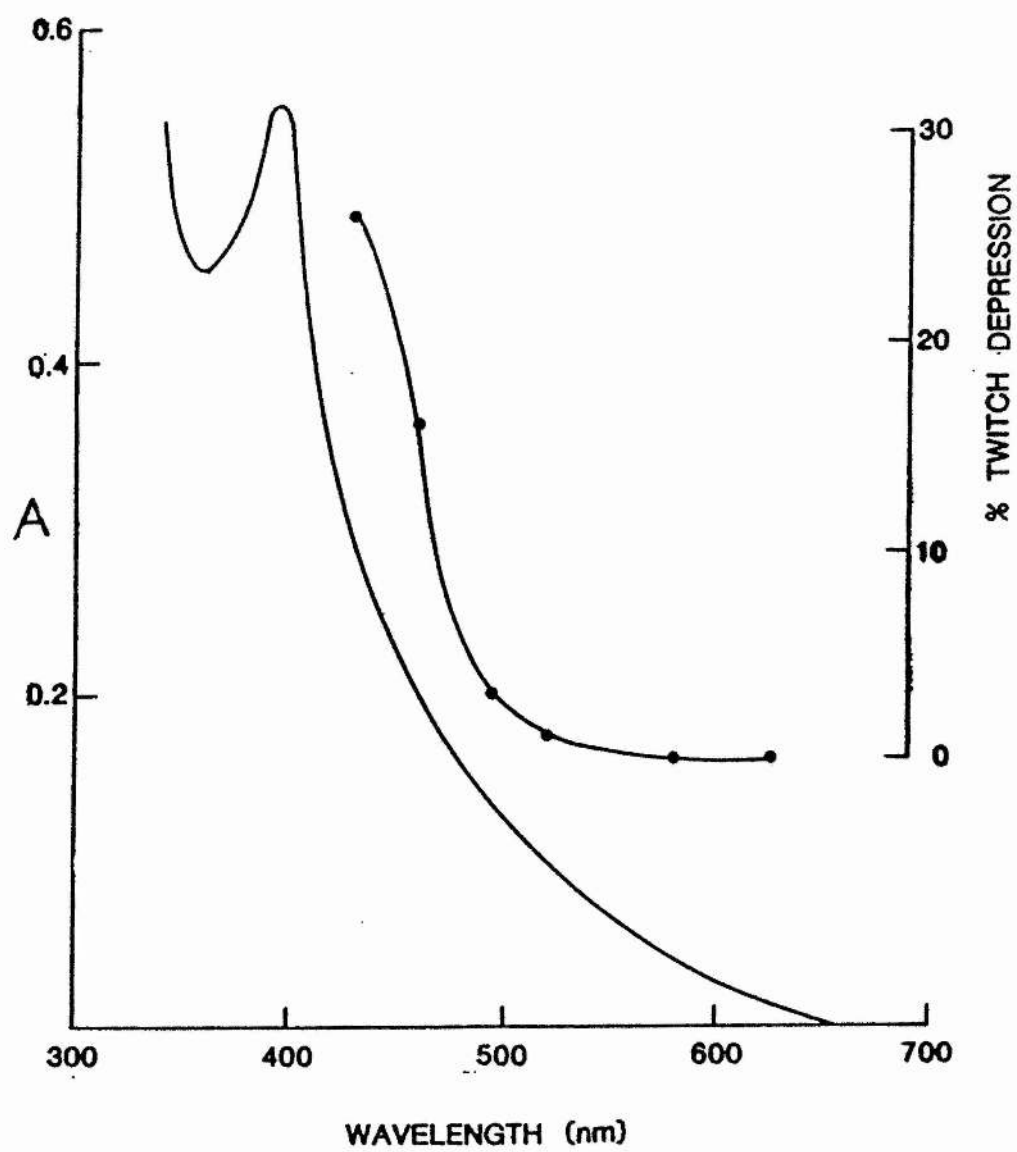
intensity. Figure 3:6 shows the extent of twitch depression, superimposed on the absorption spectra of NP. The points are plotted against the wavelength at which transmission through the filter is maximal, but most filters had a fairly wide bandwidth such that they overlapped one another. Even so, the size of the response clearly varies with wavelength, with the blue end of the spectrum being much more effective at depressing the twitch. The absolute size of the maximal response measured here is very small due to the low intensity of light used in order to keep it constant at all wavelengths. The sharp increase in the size of the response seen at shorter wavelengths ( $<500\text{nm}$ ) roughly parallels the increase in absorption of NP.



## Chapter 3

### Figure 3:6

The wavelength of the incident light was changed by using a combination of neutral density and coloured filters to keep the total intensity constant. The extent of the twitch depression caused by  $10^{-2}$  M NP, varied with the wavelength of illumination as shown in the right hand curve. The left hand curve shows the absorption spectrum of NP plotted along the same X-axis as the response curve. The increased depression of the twitch correlates with an increased absorbance of the drug. It should be noted that the filters are plotted at the wavelengths of their peak transmission but the spectral spread of each filter is large and overlapping.



Discussion

The most important conclusion to be drawn from these results is that NP must first be exposed to light before it can exert any effect on the twitch. This was an unexpected result in view of the previous report of a strong negative inotropic action of NP on frog ventricle (Flitney, Moshiri & Singh; 1980a,b). There are however a number of important differences between these two studies. Flitney, Moshiri & Singh (1980) showed a greater rate and level of depression of the twitch for a given concentration of NP; eg. at  $10^{-2}M$ , the twitch was reduced to 20% of its control within 2 min. They were also able to elicit responses at NP concentrations 100 fold less than the threshold concentration seen in these experiments. These differences can however be explained by the different experimental conditions.

The reason why NP has any effect at all is because although the authors were aware of the photosensitivity of NP, they did not realise how extremely sensitive it is. Their experimental set up had a constant reservoir of NP which was gravity fed to superfuse the half ventricle and then re-circulated back to the reservoir via a peristaltic pump. The flow rate was 100ml/min, and so the 1 litre reservoir solution was being completely circulated every 10 min. Although most of this tubing was covered with aluminium foil, the solution was nevertheless exposed to the ambient light just before and during the time when it was in contact with the preparation. This repeated exposure to light was presumably sufficient to elicit a response. Evidence for this

comes from their observation that a second application of the same solution potentiated both the rate and extent of depression of the twitch. Presumably, some photolytic product accumulated in their solution, so that a second exposure resulted in a more rapid and greater response.

The greater efficacy of NP seen in the previous experiments may be due to two reasons.

1. The half ventricle preparation was allowed to become hypodynamic before the effects of the drug was tested. In our experiments this was prevented from happening by adding pyruvate to the ringer. It is possible that the depressant effect of NP is exaggerated on an already weakened preparation, although this hypothesis has not been tested.

2. Flitney, Moshiri & Singh (1980) may have inadvertently exposed their solutions to a greater total amount of light. The present results show that increasing the intensity of illumination enhances its ability of NP to decrease the twitch. If the total illumination of NP in the previous studies was greater than in these experiments, then this might explain the greater sensitivity to NP. This does not seem likely since solutions which were pre-exposed for 1hr to a high intensity of light, produced a smaller response. A possible explanation for this discrepancy may be that the active agent formed during exposure of the NP soln. may be unstable. If this were the case then much of the physiologically active agent may be lost. On the other hand, if the active agent were instead generated close to its site of action as is the case when exposing solutions in the muscle chamber, then it would have a greater effect. In other words,

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the total amount of exposure may be less important than where the exposure takes place in relation to the muscle.

The results from this series of experiments can be summarised as follows: Firstly, the nitroprusside anion per se has no effect on twitch contractures of frog ventricular muscle. Secondly, exposure of NP to light elicits a response that is directly related to the NP concentration and the intensity of illumination, but inversely related to the wavelength. Thirdly, the rate and extent of depression of the twitch are both influenced by the time interval between exposure of NP to light and its delivery to the muscle.

Taken together, these results suggest that i. photolysis of NP generates a physiologically active species that can depress contractility, and ii. the photolytic product in question is relatively unstable in solution.

## CHAPTER 4.

An investigation into the nature of the  
photochemical transformation of nitroprusside.

### Introduction

It is clear from the preceding chapter that nitroprusside (NP) undergoes a photochemical reaction that transforms it from being physiologically inert to active. The nature of this change is investigated in this chapter.

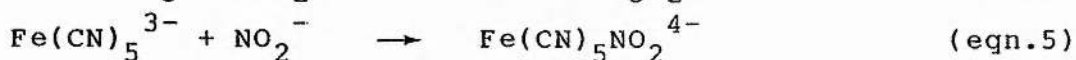
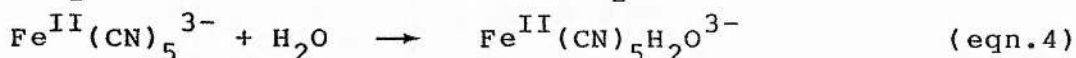
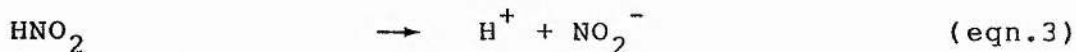
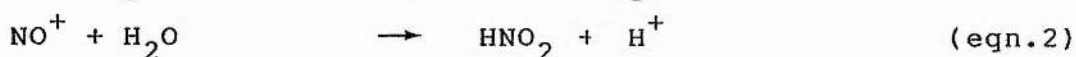
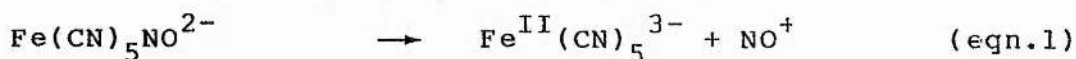
Sodium nitroprusside in solution is extremely photosensitive and undergoes a variety of reactions when exposed to light. Even in the crystalline state, NP is reported to be light-sensitive (Rucki 1977). This may however, be due to small amounts of moisture facilitating breakdown, as solid NP kept in closed amber vials, did not show any decomposition after a year. Moreover, solutions of NP in water are stable for at least two years both at room temperature, and at 4°C provided they are kept in the dark (Vesey and Batistoni 1977).

Descriptions in the literature of the photolytic reaction sequences and products of NP are as numerous as they are contradictory. It appears that the final products of photolysis depend on the solvent used, the wavelength of the light, the oxygen content of the solution and the pH. Unbuffered solutions of NP in water irradiated by either sunlight, fluorescent light, tungsten light or wavelengths greater than 300nm, resulted in the formation of Prussian blue ( $\text{Na Fe}^{\text{III}}\text{Fe}^{\text{II}}(\text{CN})_6$ ), nitric oxide (NO), hydrogen cyanide (HCN) and free cyanide ( $\text{CN}^-$ ) (Arnold, Longnecker, & Epstein, 1984; Vesey and Batistoni, 1977; Rucki, 1977; Frank, Johnson & Rubin, 1976; Wolfe and Swinehart, 1975). In buffered solutions (pH 6) nitric oxide and aquapentacyanoferrate



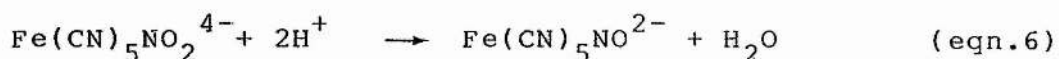
( $\text{Fe}^{\text{III}}(\text{CN})_5\text{H}_2\text{O}^{2-}$ ) are the only decomposition products observed over photolysis periods of up to 16 hr (Wolfe & Swinehart, 1975). This agrees with other studies that the observed increase in the absorption spectra at 394nm during photolysis of SNP is due to production of aquapentacyanoferrate(III) as the primary photolytic product (Espenson & Wolenuk, 1972; Arnold et al, 1977; Rucki, 1977 ).

The other primary photolytic products of nitroprusside and the photochemical pathways involved remain contentious issues. Mitra, Jain, Banerjee & Chari (1963) proposed the following reaction sequence to explain their observations ;

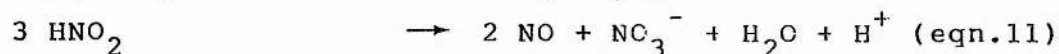
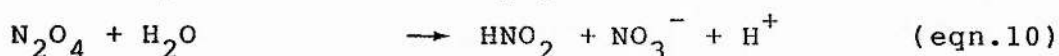
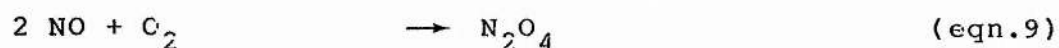
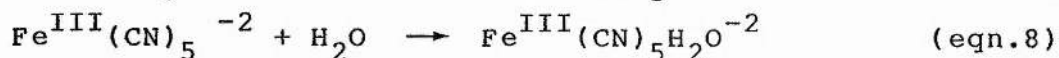
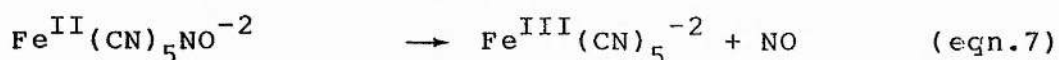


They postulated that the primary photochemical reaction (eqn.1) liberates the nitrosyl cation ( $\text{NO}^+$ ) from nitroprusside. The formation and dissociation of nitrous acid (eqns.2&3) has been suggested to be responsible for the observed decrease in pH when NP is photolysed in unbuffered solution. Detection of the nitrite ion has supported this theory. The formation of  $\text{Fe}^{\text{II}}(\text{CN})_5\text{H}_2\text{O}^{3-}$  (eqn.4) is reported to be responsible for the increased absorbance at 394nm. There is however disagreement on this point, as others have shown that the species with maximum absorbance at 394nm is  $\text{Fe}^{\text{III}}(\text{CN})_5\text{H}_2\text{O}^{2-}$  (Espenson & Wolenuk, 1972). Further evidence for this pathway comes from the observation that a photolysed solution of NP regains its original pH if left in the dark

(Mitra et al, 1972). This is consistent with the equilibrium shown in equation 6;



Wolfe and Swinehart (1975) after further investigations of these phenomena have criticised this hypothesis. They found that the large pH decrease during photolysis was oxygen dependent, and that the photolysed solution did not recover its original pH if left in the dark. The size of the pH drop in oxygenated solutions compared to that in deoxygenated solutions cannot be accounted for by Mitra's scheme. Dissociation of nitrous acid (eqn.2 & 3) could only increase the hydrogen ion content by a factor of 2, not the tenfold increase observed. This has led these authors to propose another pathway, with liberation of nitric oxide (NO), rather than  $\text{NO}^+$ , as the primary photochemical reaction.



On this scheme, the aquapentacyanoferrate(III) species (eqn. 8) is responsible for the increased absorbance at 394 nm (Espenson & Wolenuk, 1972). The production of NO gas was confirmed by mass spectrometry (Wolfe & Swinehart, 1975). The decrease in pH can be accounted for by the sequence of chemical reactions given in equations 9-11. These reactions not only increase the hydrogen ion concentration by the required amount, but also account for the oxygen consumption

during photolysis (Wolfe & Swinehart, 1975). The disproportionation of dinitrogen tetroxide (eqn.10) could also be responsible for nitrite formation in air saturated solutions of photolyzed NP. Reports of nitrite production in deoxygenated NP solutions may be due to the use of air saturated reagents (Mitra et al, 1972).

Leeuwenkamp, (1984) in his review suggested that the two pathways might be integrated in view of the results reported by Jarzynowski et al (1977). They found that the mechanism of photolysis of NP was dependent on the wavelength of irradiation. Irradiation at 498nm wavelength (band I) did not cause any photodegradation, and whereas 396nm (band II) irradiation resulted in hydration (eqn. 1&4), 265nm or lower (band IV-VI) irradiation resulted in photo-oxidation and exchange of NO by H<sub>2</sub>O (eqn. 7&8). These wavelengths correspond to the absorption bands of NP and are interpreted as being due to specific transitions in the electron distribution of the molecule. Different irradiation wavelengths therefore change the molecular orbital distribution in different ways and so result in different breakdown products. The overlapping of photochemically active bands (band III overlaps with band II & IV) means that both pathways can occur simultaneously. However, this interpretation does not agree with the MO level diagram according to the self-consistent charge and configuration (SCCC) method (see table 1:1)(Swinehart, 1967; Wolfe & Swinehart, 1975; Leeuwenkamp et al, 1984). According to this diagram, longer wavelength irradiation (>390nm : bands I & II) result specifically in nitric oxide cleavage (not nitrosyl cation), whereas shorter wavelengths cause relatively

non-specific metal-ligand bond cleavage (Wolfe & Swinehart, 1975). Any polychromatic (and even some monochromatic) irradiation would result in a mixture of breakdown products, depending on which photochemically active bands it covered and the relative intensity at each. This may account for some of the variation in the reported products of nitroprusside photolysis.

The preceding account is only concerned with the primary photochemical reactions in the photolysis of nitroprusside. Numerous secondary reactions have been described in the literature.  $\text{Aq}(\text{II or III})$ , undergoes rapid equilibrium with  $\text{Fe}_2^{\text{II}}(\text{CN})_{10}^{6-}$  or  $\text{Fe}_2^{\text{III}}(\text{CN})_{10}^{4-}$  respectively (Rucki, 1977). Oxidation of  $\text{Aq}(\text{II})^{3-}$  to  $\text{Aq}(\text{III})^{2-}$  has been reported and is thought to be responsible for the orange to blue colour change of a photolysed solution of NP (Rucki, 1977; Van Loenen & Hofs-Kemper, 1978). Conversion of  $\text{Aq}(\text{II or III})$  to hexacyanoferrates (II or III) has also been reported, with the release of  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{CN}^-$  and ultimately forming prussian blue (Leeuwenkamp et al, 1984; Van Loenen & Hofs-Kemper, 1978). In the presence of  $\text{Fe}^{2+}$  ions and oxygen  $\text{Aq}$  degrades to form numerous dimetallic ionic species (Hofs-Kemper, 1978). In solutions with  $\text{pH} > 7$   $\text{Aq}(3-)$  forms  $\text{Aq}(4-)$  which further photodegrades to release cyanide and produce aquatetracyanoferrate(4-) (Van Loenen & Hofs-Kemper, 1978). The secondary reactions are therefore numerous and complex but can only occur after the Nitroprusside is exposed to light. This is because after photolysis, the change in the oxidation state of the central iron weakens the bond strength to the surrounding cyanide groups, such that they become susceptible

to substitution reactions. The stable NP molecule is then transformed into the labile Aq molecule which is then able to participate in numerous reactions. Note that it is only during these secondary reactions that cyanide is liberated. This is relevant in the clinical use of NP as it has been reported that photodegradation before infusion may be responsible for the in vivo release of cyanide ( Bisset, Butler, Glidewell, Reglinski, 1981).

The results from the previous chapter show that nitroprusside must first be exposed to light before it can exert any negative inotropic action on frog ventricle. These results were of a preliminary nature as neither the method of exposure nor the interval between exposure and application were carefully controlled. Illuminating the drug solution while it is bathing the muscle is not altogether satisfactory as the degree of exposure would vary. Most of the solution being exposed would be flowing past the trabeculum in the chamber and so would only be briefly irradiated before being washed away without exerting any effect. NP in the locality of the trabeculum, especially that 'trapped' within the intercellular clefts, would be exposed for longer and thus exert a greater effect. The effective concentration of photodegraded NP could not therefore be easily controlled. Since the muscle would also be illuminated then the light could be affecting it directly. Although control experiments showed no effect of illuminating the muscle in Ringer solution, the light could be affecting one or more steps in the chain of events leading to a response. Indeed there is evidence of a direct effect of long UV light (350-450nm) on

## Chapter 4

crude soluble guanylate cyclase, the enzyme thought to stimulated by NP (Karlsson, Axelsson & Anderson, 1985). In order to study the effect of light on NP alone, solutions must be illuminated away from the muscle and then applied to it. This was attempted in the 'pre-exposure' experiments described in the previous chapter, but it led to difficulties in the time between exposure and application. The remedy adopted here was to illuminate NP solutions en route to the muscle chamber, such that photolysis takes place just before the perfusate reaches the muscle. This was achieved using the apparatus described below and shown in figure 4:1.

A laser was used to illuminate the solution in this tube for two reasons (i) the small beam allowed light to be accurately directed down the tube; (ii) it allowed an exact quantitative measure of the intensity and wavelength of the incident light. This enabled a detailed study to be made of the effect of changing both the intensity and wavelength of light on the physiological responses.

The photolysis of NP was monitored in these experiments by measuring the increase in absorbance at 395nm before and after exposure to light. This is due to the formation of Aq(III) which has an extinction coefficient at 395nm of  $743 \text{ M}^{-1}\text{cm}^{-1}$  as compared to only  $20.4 \text{ M}^{-1}\text{cm}^{-1}$  for NP. The increase in absorbance measured at this wavelength is therefore proportional to the concentration of Aq produced.



Methods

This section deals only with the method used to illuminate the NP solution, as the procedure for recording twitch contractions was described previously.

The apparatus used is shown in figure 4:1. Exposure of solutions to laser light was achieved by passing them through a curved glass tube, fitted with a quartz glass window and outlet at one end, and which tapered away to an inlet at the other. The outer walls of the tube were silvered such that light entering through the window was totally internally reflected along its length. The perfusate, selected by means of the six way tap, flowed directly into the glass tube where it was then exposed to laser light entering through the quartz window. The volume of the tube was 10ml and the flow rate was adjusted to around 10ml/min. such that the total exposure time was 1 minute. On leaving the tube the exposed solution flowed directly into the muscle chamber through a small bore (1mm) connecting pipe. This kept the dead space to a minimum (0.5ml) and ensured that the time interval between exposure to light and reaching the muscle was as small as possible (3sec.). The laser was a 5W argon-ion laser (Spectra-Physics; model 168-09) which allowed the choice of any one of 10 discrete wavelengths. The orientation of the laser was critical, in that the beam of light had to be directed into the middle of the window. This ensured that the fastest flowing solution in the centre of the tube was properly illuminated and that reflection off the end window was minimal (<4%). The laser beam was scattered by the solution and



reflected continuously off the mirrored surface of the tube, such that it became diffused throughout the lumen of the tube.

All the experimental procedures were performed in darkness, the only form of illumination being red light from either a small hand torch or a darkroom safe lamp. The apparatus was enclosed in an aluminium box to prevent stray light entering and to ensure that the solution was only exposed to laser light during its passage to the chamber.

The degree of photolysis was estimated by monitoring the quantity of aquapentacyanoferrate (Aq) formed. This was done by measuring the absorbance at 395nm of an exposed NP solution against an unexposed NP solution. A sample of NP solution was collected after it had flowed through both the exposure tube and muscle chamber and this was read against an aliquot of the same solution that had been kept in the dark. The increase in absorbance at 395nm wavelength is directly related to the concentration of Aq formed during photolysis.

An illustrative example is shown in figure 4:2. The two superimposed traces show the absorption spectra of 5mM nitroprusside, read against distilled water, before (bottom trace) and after (top trace) exposure to 20mW 457nm laser light in the exposure tube. In this example, the flow rate through the exposure tube was  $10.5 \text{ ml.min}^{-1}$ , the difference between the two spectra at 395nm ( $= 0.322 \text{ A}$ ), normally obtained directly by reading exposed NP against an unexposed sample, is equivalent to an Aq concentration of  $43.3 \mu\text{M}$ .

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Figure 4:1

C - Muscle chamber  
E - Exposure tube  
Q - Quartz glass window  
T - six way tap  
L - Argon ion Laser  
 $\lambda$  - Laser beam  
W - to waste

The required perfusate selected by the six way tap (T), flowed in the direction of the arrows through the exposure tube (E) into the muscle chamber (C) and then to waste (W). A beam of light ( $\lambda$ ) from the laser (L), was directed into the exposure tube through a quartz glass window (Q) where it was diffused by reflecting off the mirrored walls of the tube. This ensured that the perfusate was completely exposed to light during its passage through the exposure tube.

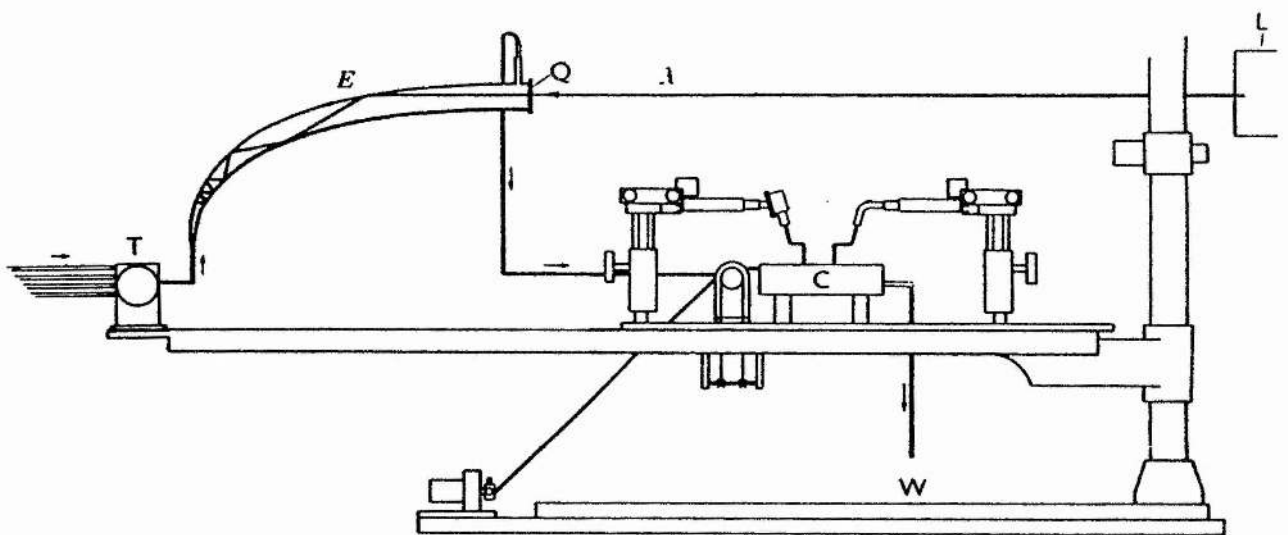
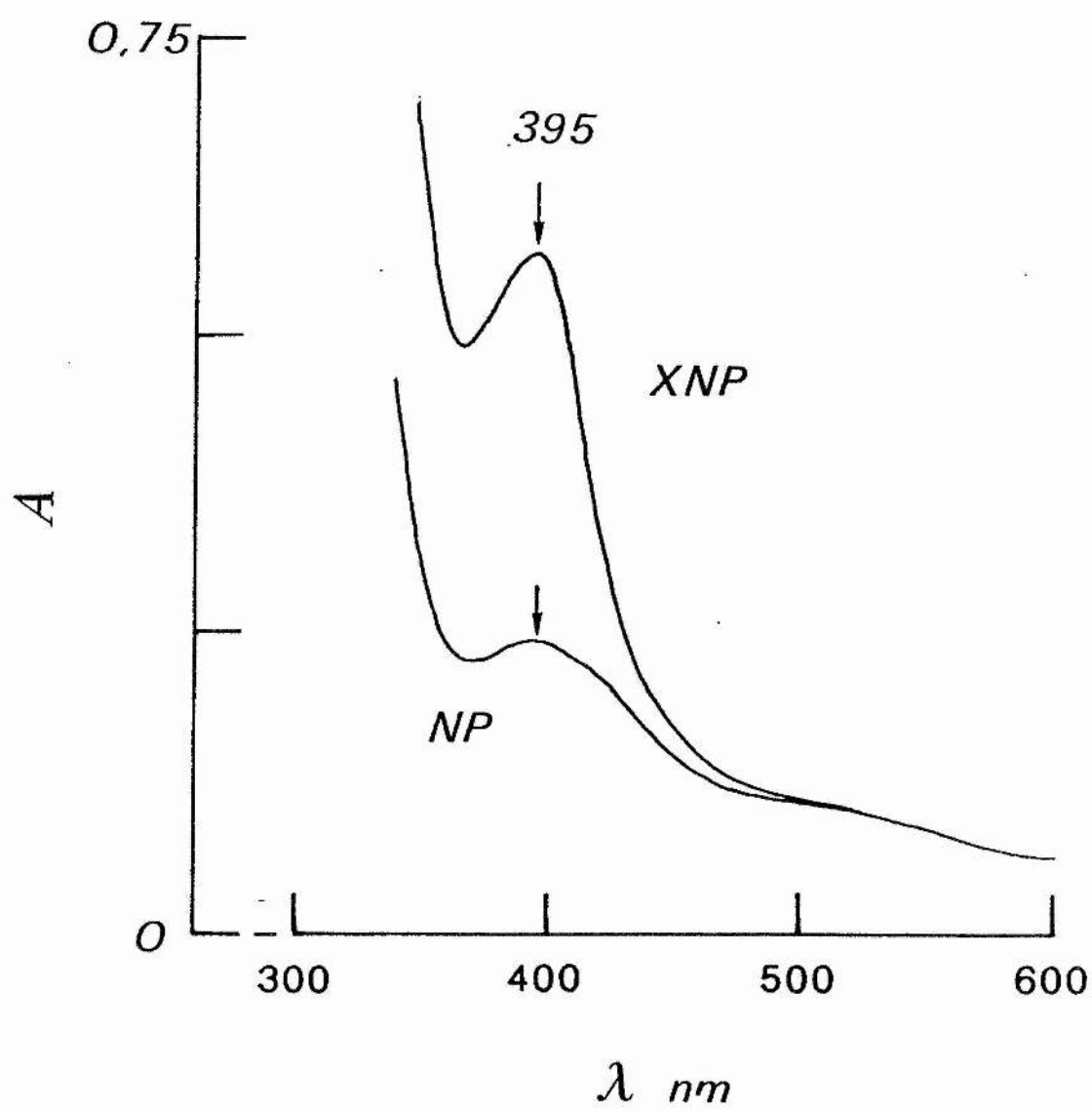


Figure 4:2

Shown here are two superimposed scanning absorption spectra of the same solution of 5 mM NP before and after exposure to 20mW of 457.9 nm laser light. The bottom trace (NP) is the absorption spectra of protected NP solution, read against distilled water as a reference. The top trace (XNP) is the same solution after exposure to 20mW of 457.9nm laser light, again read against distilled water. The increased absorbance of the exposed solution at 395nm is due to the production of aquapentacyanoferrate (Aq). This anion has an extinction coefficient at 395nm of  $743 \text{ M}^{-1}\text{cm}^{-1}$  compared to  $20.4 \text{ M}^{-1}\text{cm}^{-1}$  for NP. The concentration of Aq can therefore be determined by dividing the difference in absorbance at 395nm by the molar extinction coefficient. In this example, the concentration of Aq is 44.2  $\mu\text{M}$ .



### Results

i. The effect of Nitroprusside that was totally protected from light on the twitch tension.

As in the earlier experiments, Ringer's solutions containing nitroprusside (NP) that had been protected from exposure to light failed to have any effect on the twitch, even at concentrations of  $10^{-2}M$ . Examples of this are shown in figures 4:3 & 4:4 where the perfusate was changed from normal Ringer's solution to one containing either  $10^{-3}M$  NP (fig. 4:3) or  $10^{-2}M$  NP (fig. 4:4), neither of which had any effect on peak twitch tension.

ii. Effects of changing the wavelength and intensity of illumination.

On exposing the solution to laser light, the contractile performance of the trabeculum further downstream was impaired. This result shows that the response is not due to any direct effect of light on the muscle, but is instead a consequence of the photolysis of NP. The decrease in twitch tension begins soon after the laser is switched on and reaches a steady level in a time which is comparable to that seen on exposing the perfusate in the muscle chamber (<10min). Presumably, this is because photolysis occurs close to the muscle.

The results of these experiments confirm that at any given wavelength, the effect on the twitch is proportional to the intensity of illumination. Figure 4:4 shows a response in which the light intensity is increased from 3mW to 20mW during

Figure 4:3

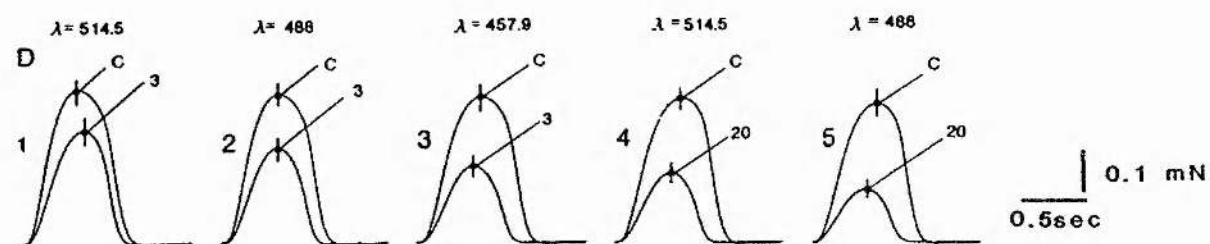
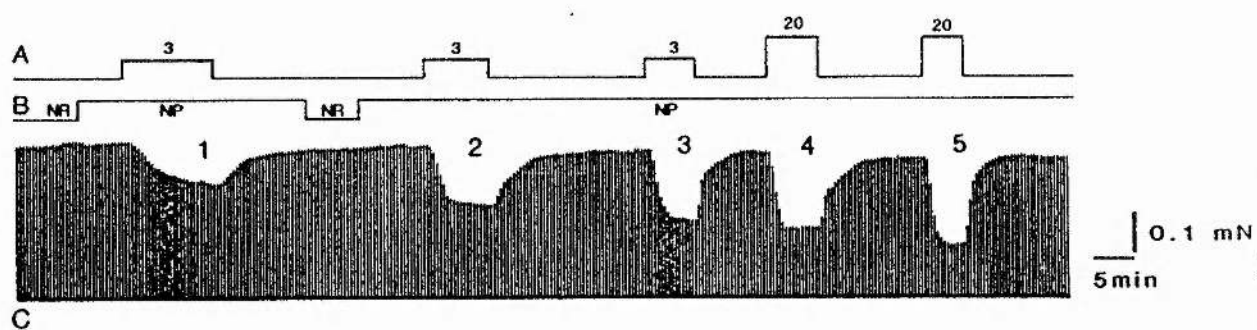
A - Panel showing pattern of illumination of exposure tube by laser. Upward defection of bar indicating start and intensity of illumination, downward deflection of bar showing end of illumination. Intensity shown above bar in mW.

B - Panel showing sequence of changeover of perfusates. NR = normal ringer; NP = ringer containing  $10^{-3}$ M nitroprusside.

C - Computer recordings of twitch height against time. Each vertical bar shows the active tension generated by every fifth twitch (i.e. time interval between bars is 25 sec ). The timescale and force produced are indicated by the scale bars on the right.

D - Steady state isometric twitches before (top traces) and after exposure (bottom traces). The timescale and force values are indicated by the scale bars to the right.

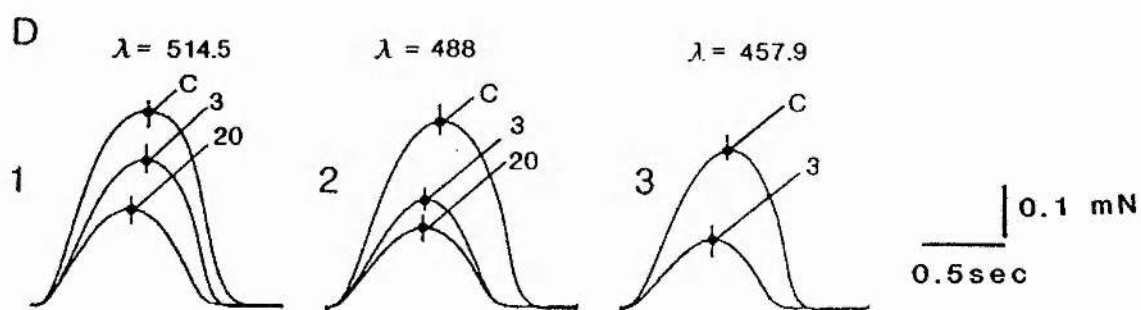
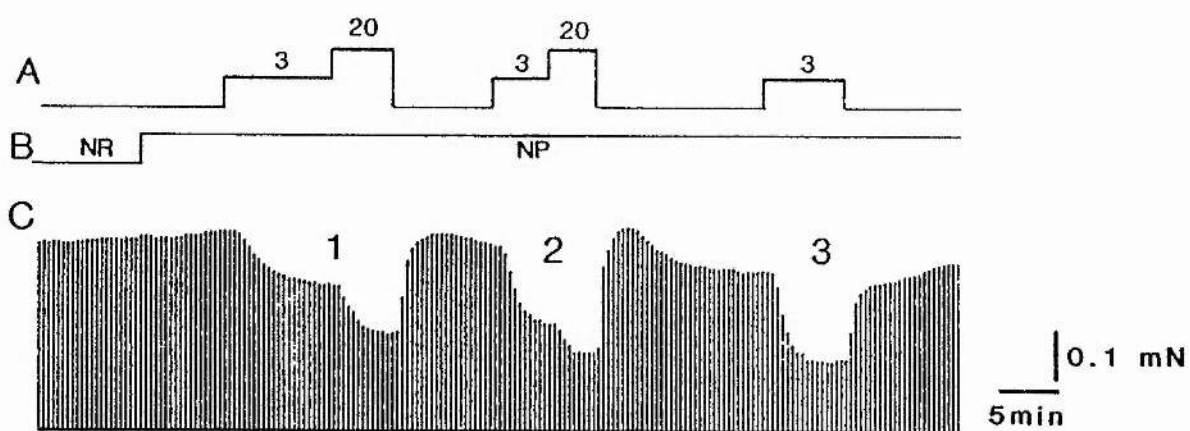




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### Figure 4:4

The panels in this figure are similar to those in figure 4:3, showing the negative inotropic action of  $10^{-2}$ M NP after exposure to various intensities and wavelengths of light.



a single exposure. Peak twitch tension is reduced to a constant level by 3mW and then is further reduced when the intensity is increased to 20mW.

The results from the previous chapter also showed that the degree of depression of the twitch is inversely related to wavelength. This was studied in more detail using three different wavelengths: 514.5nm (green light), 488nm (blue/green light) and 457.9nm (violet light). Some responses are shown in figure 4:3. At a constant intensity of illumination, either 3mW (1,2 &3) or 20mW (4&5), the depression of the twitch increases with decreasing wavelength. The difference in sensitivity amounts to a factor of almost 7 over the range studied: thus 20mW of light at 514.5nm is required to produce an equivalent twitch depression to only 3mW at 457.9nm.

iii. The effects of exposed NP on the shape of the twitch.

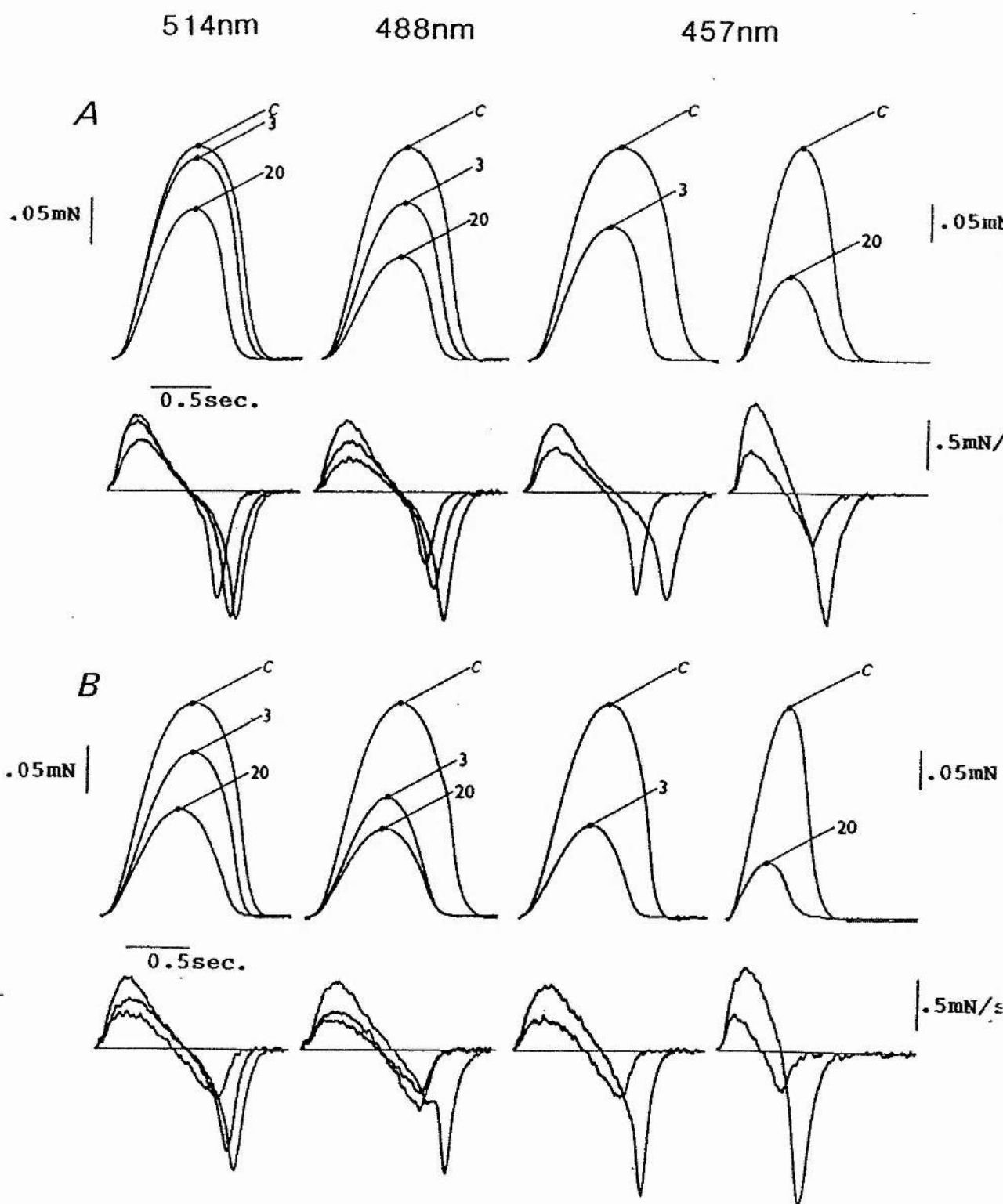
The decrease in peak tension induced by exposed NP, is accompanied by a decrease in both the time to peak (TTP) and maximum rate of rise ( $dp/dt$ ) of tension. To illustrate this effect, representative steady state twitches produced by two different NP concentrations after each illumination condition, are shown superimposed on the control twitch (figure 4:5). The differentiated tension signals are shown below on the same time scale. These show more clearly the shortening in the TTP (where the signal crosses the zero line) and the decrease in the  $dp/dt$  (decrease in the peak of the differentiated signal).

It can be seen that the moderate reductions in peak tension, produced at low light intensities, lower NP

Figure 4:5

The change in the twitch shape for a preparation exposed to  $10^{-4}\text{M}$  (panel A) or  $10^{-2}\text{M}$  (panel B), NP before and during illumination at varying intensities and wavelengths of light. The top half of each panel shows the isometric twitch recorded before (C) and after exposing NP to 3mW (middle traces) or 20mW (lower traces) laser light. Each twitch is superimposed on its respective preceding control twitch (C), recorded before the onset of illumination. The wavelength of light used is shown above each column. Two separate columns are required for 457nm light as each intensity was tested on different experiments such that the controls varied.

The bottom half of each panel shows the differentiated form of the twitches on the same time scale. This helps to illustrate the changes in twitch shape that occur. The peak of the differentiated signal corresponds to the maximum rate of rise of tension, whereas the time to peak tension corresponds to the point where the signal crosses the baseline.



concentrations and longer wavelengths, are associated with a reduction in  $dP/dt$  but with little or no change in TTP. Any further decrease in peak tension, caused by higher intensities, NP concentrations or shorter wavelengths is accompanied by reductions in both  $dP/dt$  and TTP.

#### iv. Time course of the decline in peak twitch tension.

Figure 4:6 shows the time course of the decline and recovery of the twitch after  $10^{-3}M$  NP was exposed to each of the three wavelengths at intensities of 3mW (top panel) and 20mW (bottom panel). Each trace is the averaged peak twitch tension from 5 experiments with the standard error of the mean plotted for every fifth twitch. The results show that the rate at which the twitch reaches its new steady state level is also determined by the illumination conditions. For any given intensity, the rate of decline of tension is increased by reducing the wavelength. Similarly, for a given wavelength, the rate is also increased at greater intensities of illumination.

The time course for the recovery of the twitch after illumination ceases, but while the preparation is still in NP, is similar for all three wavelengths at any given light intensity. This is shown in the legend to fig.4:6, the small differences in the half time to recovery being well within the margin of experimental error. It would appear therefore that the recovery process is not influenced by the choice of wavelength. There are differences, however, in recovery rates between the two light intensities. This does not seem to be related to the degree of depression of the twitch, as similar

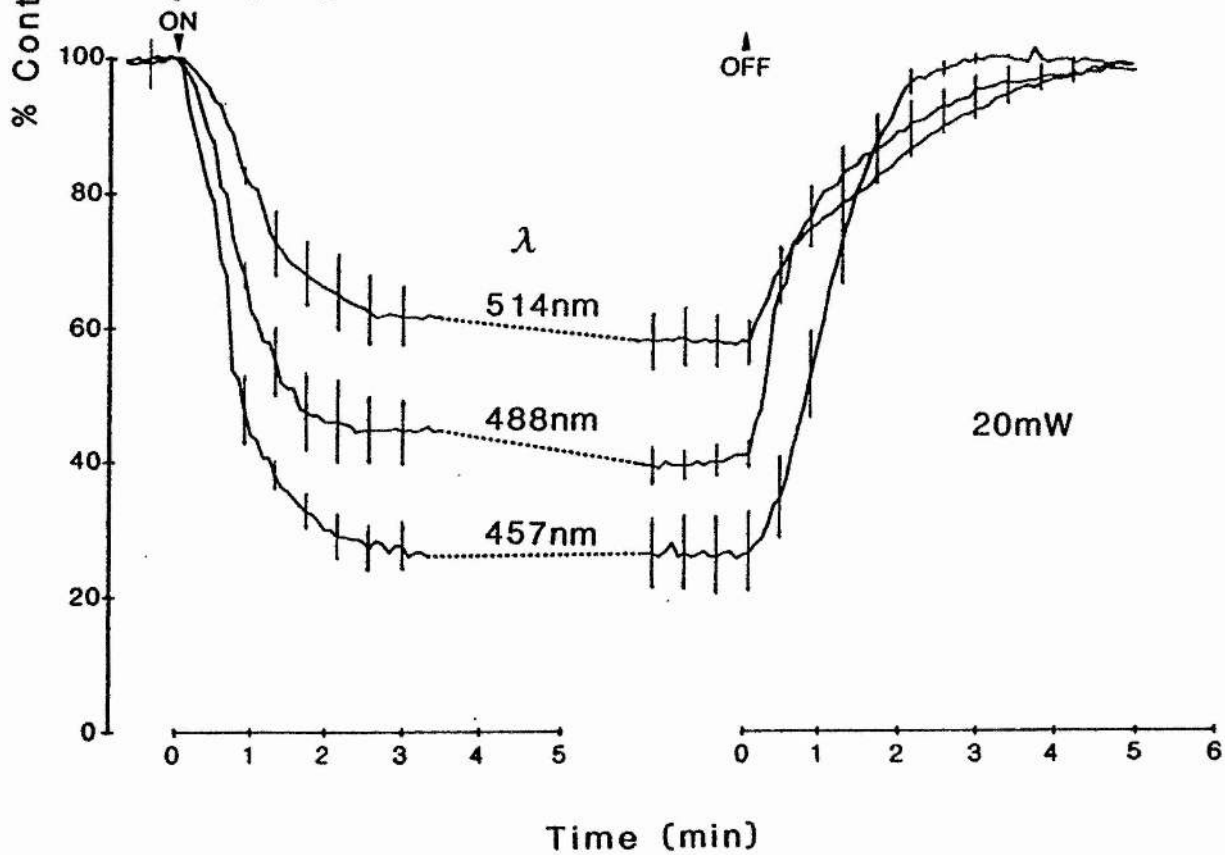
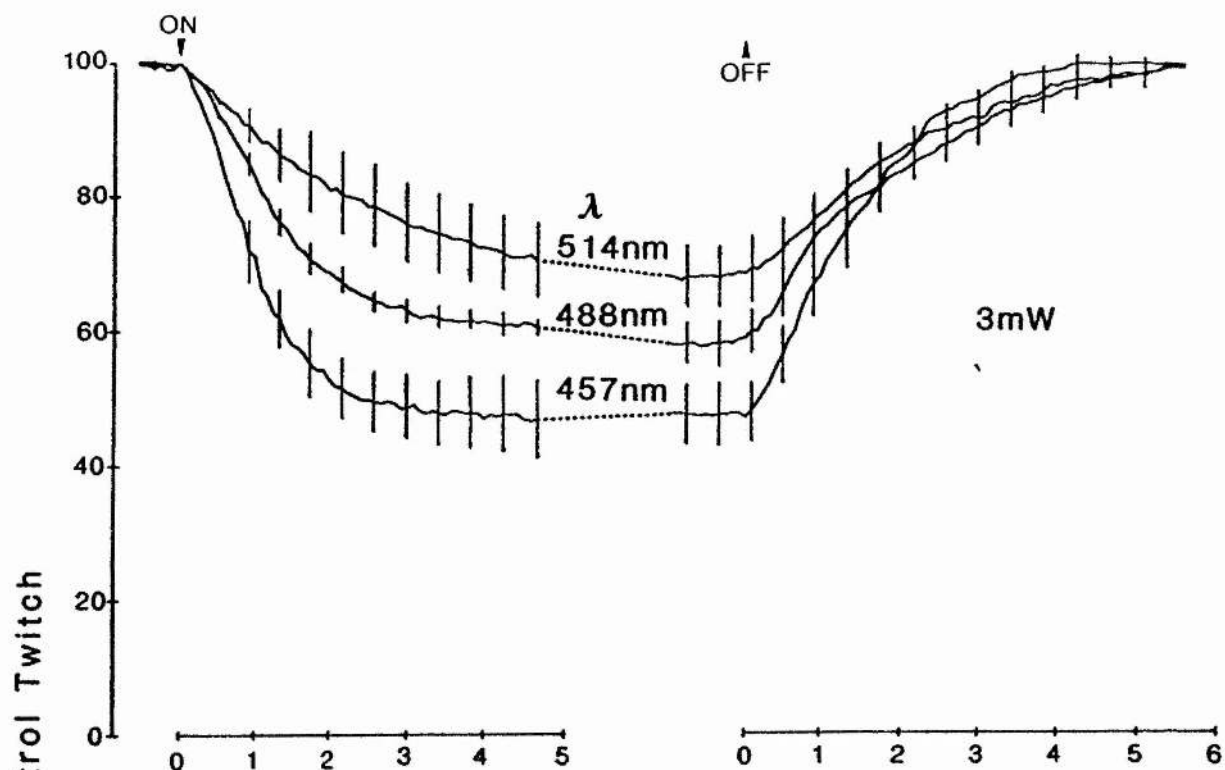
Figure 4:6

The averaged time course of the decline and recovery of the twitch after exposing  $10^{-3}$  M NP to light of 3mW (top graph) or 20mW (bottom graph) at three different wavelengths. The three wavelengths used were 514nm (top traces), 488nm (middle traces) and 457nm (bottom traces). Each trace is the averaged time course of 5 computer recordings of the peak twitch tension, normalised to the value recorded at the onset of illumination. Standard error bars are shown for every fifth twitch.

The times to a half maximum response are tabulated below;

<u>nm</u>	<u>ON (sec.)</u>		<u>OFF (sec.)</u>	
	<u>3mW</u>	<u>20mW</u>	<u>3mW</u>	<u>20mW</u>
514.5	102 + 18	69 + 5	94 + 6	63 + 10
488	72 + 6	54 + 3	81 + 9	48 + 5
457.9	54 + 5	39 + 3	78 + 10	67 + 5





responses, for example those elicited by 3mW 488nm and 20mW 514, recover at different rates. It is of interest to note that responses produced at the lower intensity show slower recovery rates. This is an indication that the overall duration of exposure may be important because the twitch took longer to reach a steady depressed state at the lower intensity.

v. Time course of the change in isometric twitch shape.

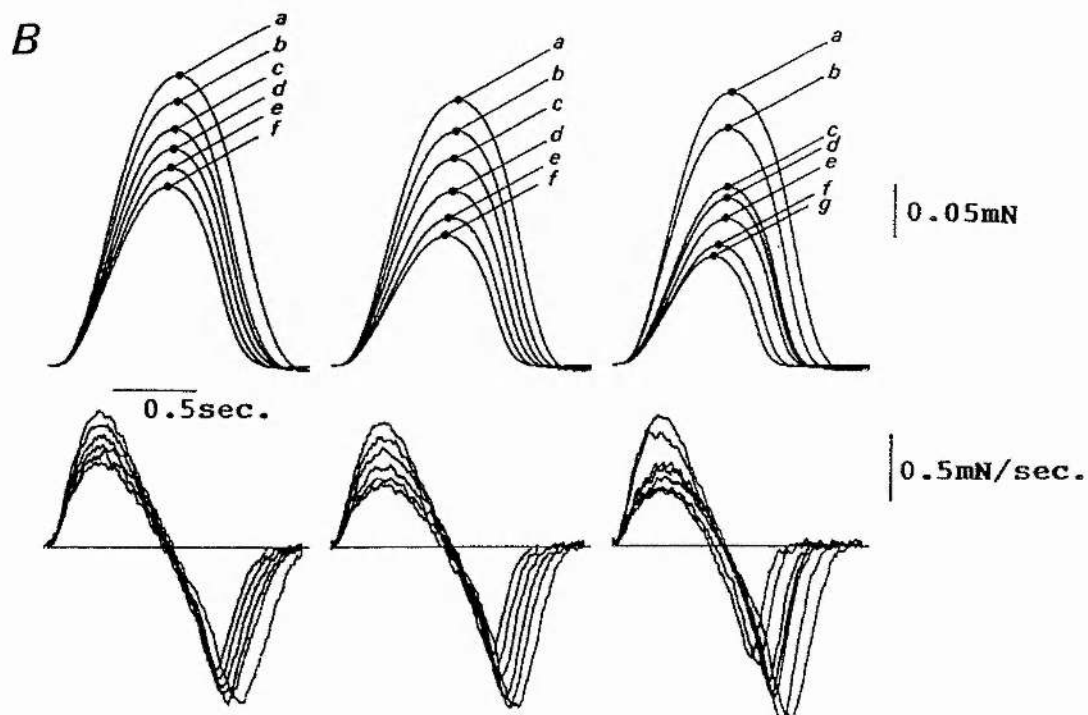
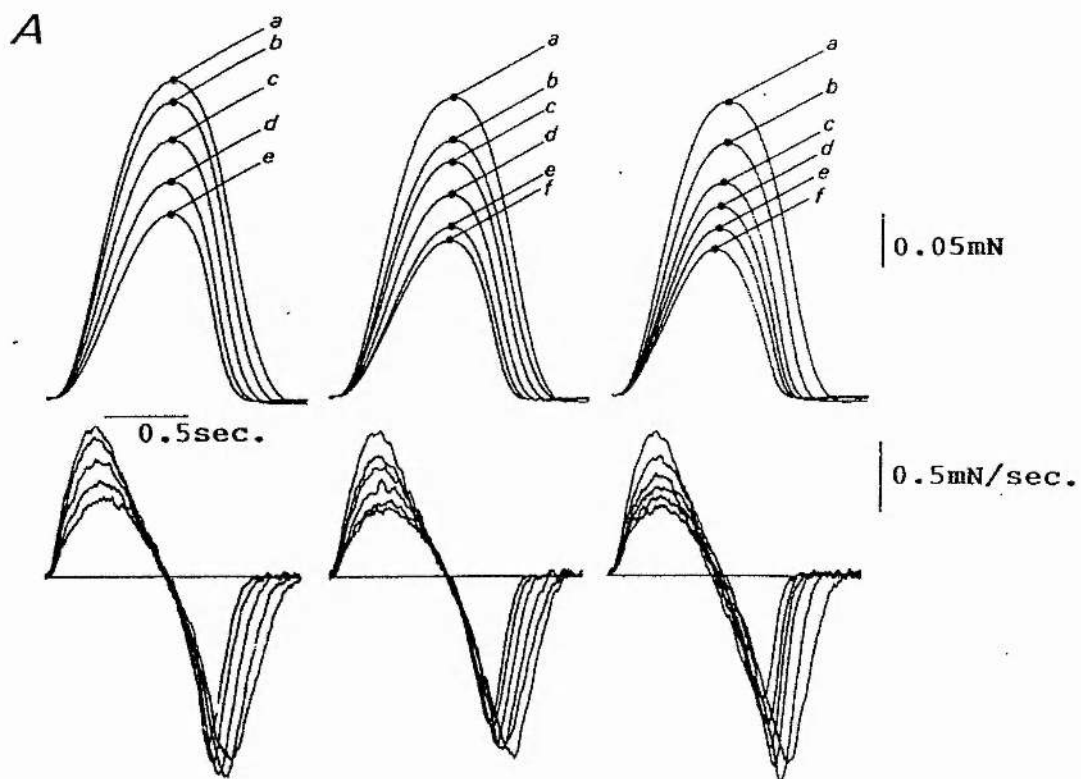
Figure 4:7 shows a series of twitches recorded during exposure to either  $10^{-4}\text{M}$  (top panel) or  $10^{-2}\text{M}$  (bottom panel) NP illuminated with 3mW of light at each of the three wavelengths. The time elapsed from the onset of illumination to each recording is listed in the legend. The corresponding differentiated twitches are shown below. Under all illumination conditions and [NP]'s, the initial decrease in peak tension is accompanied by a decrease in  $dP/dt$  but there is little or no change in TTP. However, as the duration of exposure to NP increases, the depression of the twitch becomes more pronounced, and this is associated with reductions in both  $dP/dt$  and TTP. Under conditions that cause maximal depression of the twitch (i.e.  $10^{-2}\text{M}$  NP illuminated with 20mW 457.9nm),  $dP/dt$  reaches its minimum value before peak tension. Any further reduction of the twitch is then associated with a shortening of the TTP. These results confirm that small depressions of the twitch are associated only with a decrease in the rate of tension generation, whereas larger depressions also involve a shortening of the time to peak tension.

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Figure 4:7

Isometric twitches recorded at different times after the onset of 3mw intensity illumination of either  $10^{-4}\text{M}$  (panel A) or  $10^{-2}\text{M}$  (panel B) NP. The wavelength used is shown above each column. Each panel shows superimposed twitches and the corresponding differentiated traces. These show the changes in twitch parameters more clearly. The times (in minutes) of recording each twitch after the onset of illumination are tabulated below.

	$10^{-4}\text{M}$			$10^{-2}\text{M}$		
	514	488	457	514	488	457
a	0	0	0	0	0	0
b	5.1	2.0	1.9	0.9	0.4	0.6
c	8.3	3.0	3.9	1.6	1.8	1.1
d	10.7	5.2	4.5	2.8	2.5	1.5
e	14.2	9.0	6.6	4.3	4.8	2.0
f		13.8	12.7	8.4	7.7	4.5
g			15.8			5.4



vi. Dose response curves to NP under various conditions of illumination.

All the data on the effect of exposed NP on the peak twitch tension is summarised in figure 4:8. Each log dose response curve shows the average depression of the twitch for one exposure condition over a range of [NP]'s. The curves are grouped according to intensity of illumination, 3mW on the left and 20mW on the right, while the different wavelengths are indicated by the key (see fig.4:8).

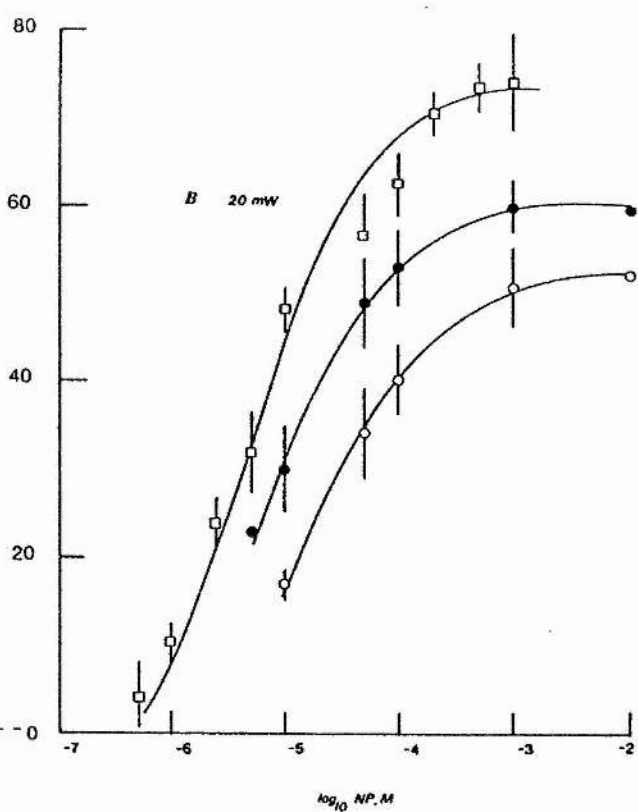
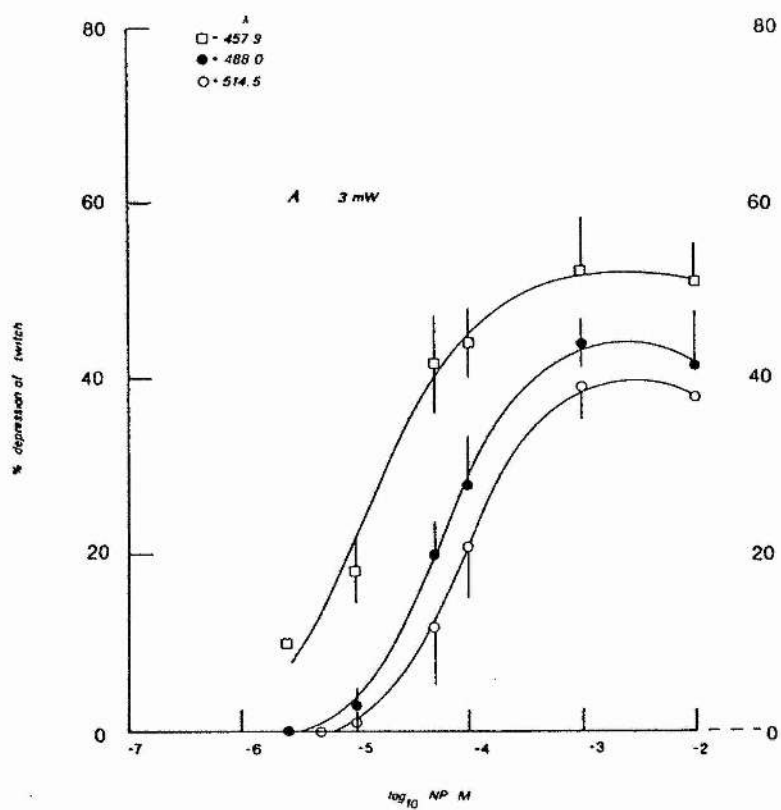
Each curve approximates to a normal sigmoidal dose response curve, reaching a plateau at [NP] of  $10^{-3}$  M. The level of the maximum response is found to depend upon the illumination conditions. It is elevated by either decreasing the wavelength or increasing the intensity of the illumination. As well as an increase in the maximal level of depression, the whole curve is shifted upward and to the left. Thus for each [NP], increasing the intensity at constant wavelength enhances the response whereas increasing the wavelength at constant intensity depresses it.

To demonstrate that this is indeed due to a shift of the dose response curve and not merely a consequence of the altered maximum response, all the data were normalised to their respective maximal responses and plotted together in figure 4:9. At any one intensity, either 3mW (C-E-F) or 20mW (A-B-D), the log dose response curve is shifted to the right with increasing wavelength. Conversely at any one wavelength the dose-response curve is shifted to the left by an increase in intensity (ie. C-A, E-B or F-D). It is clear from this that the most effective illumination condition is 20mW at 457nm and

Figure 4:8

A. Log dose response curves to NP exposed to 3mW of light at three different wavelengths.

B. Log dose response curves to the same wavelengths but at the higher intensity of 20mW. The wavelengths used were 514 nm (bottom curves), 488 nm (middle curves) and 457 nm (top curves).

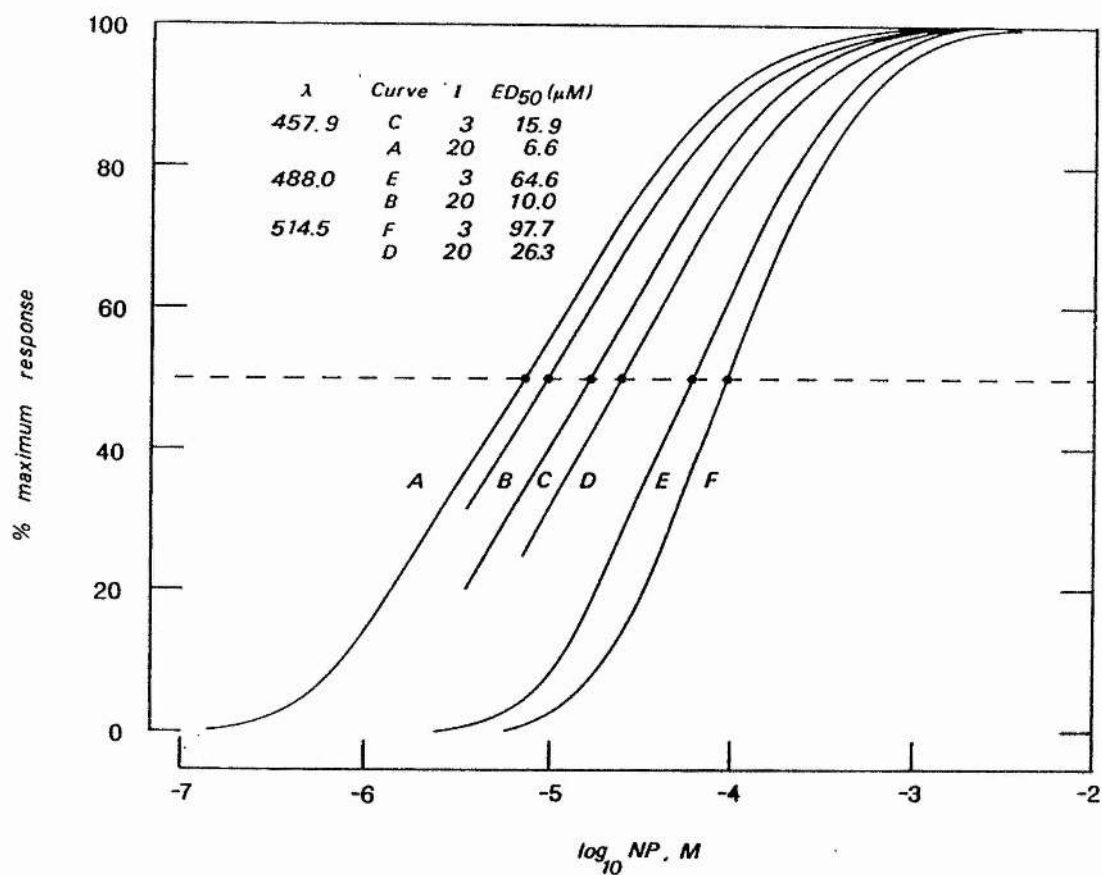


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### Figure 4:9

The log dose response curves shown in figure 4:8 were normalised to their respective maximum responses. This shows more clearly the shift in the position of the curve under the various lighting conditions. The measured E.D.<sub>50</sub> values for each illumination condition are shown in the inserted table.





the least effective is 3mW at 514nm.

vii. Log dose response curves for the change in twitch shape parameters

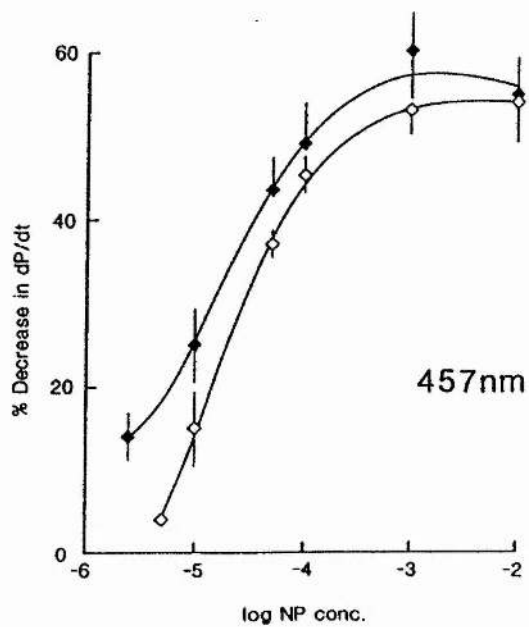
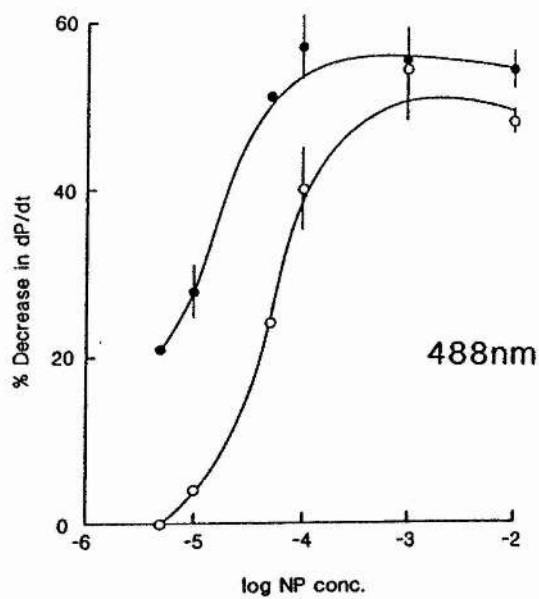
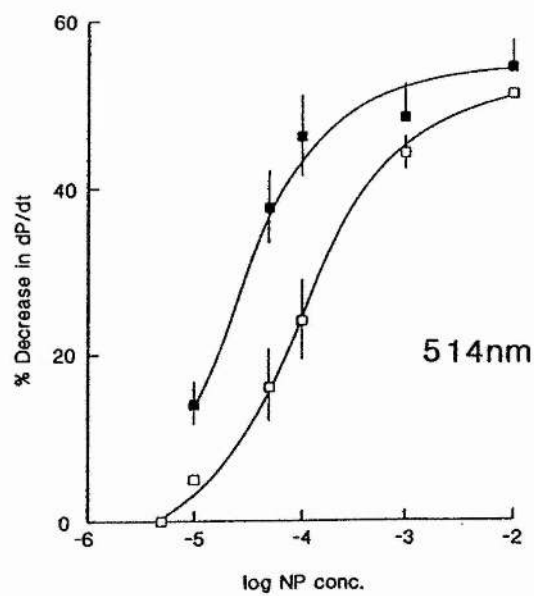
An analysis was made of the effect of changing the illumination conditions and [NP] on twitch shape. The resultant log dose response curves for the effects on  $dP/dt$  and on TTP are shown in figures 4:10 and 4:11 respectively.

The decrease in  $dP/dt$  is dependent on both the NP concentration and illumination condition. All of the curves are sigmoidal, with a maximum depression of 55%. Note that this maximum is independent of the illumination condition; the more efficient illumination conditions (i.e. 20mW 457/488nm) reach this level at lower [NP]'s than less efficient ones (i.e. 3mW 514nm), so that the relative position of the curve moves to the left. Hence, increasing the intensity of illumination produces a leftward shift of the curve whereas increasing the wavelength moves it to the right. It is of interest to note that both the 20mW 457nm and 20mW 488nm relationships are nearly superimposable. Presumably both conditions are sufficient to produce a maximal reduction in  $dP/dt$ .

In comparison, the shortening of TTP (fig. 4:11) is only appreciable at higher [NP]'s. The dose response curves show that both the slope and maximum degree of reduction in TTP are determined by the illumination conditions. More effective illumination conditions do not shift the position of the curve but instead steepen the relationship and increase the size of the maximum response. This explains why although

Figure 4:10

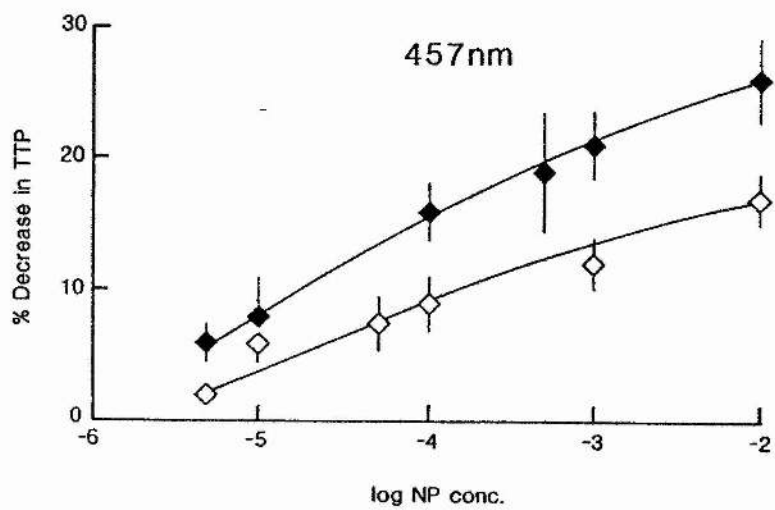
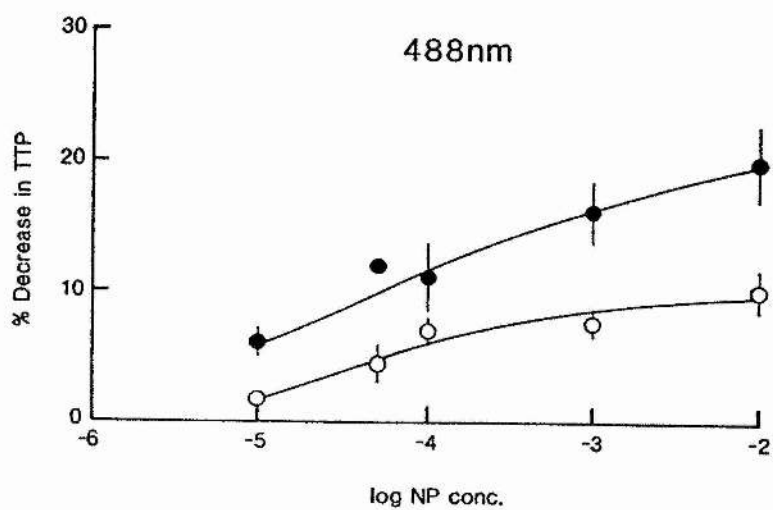
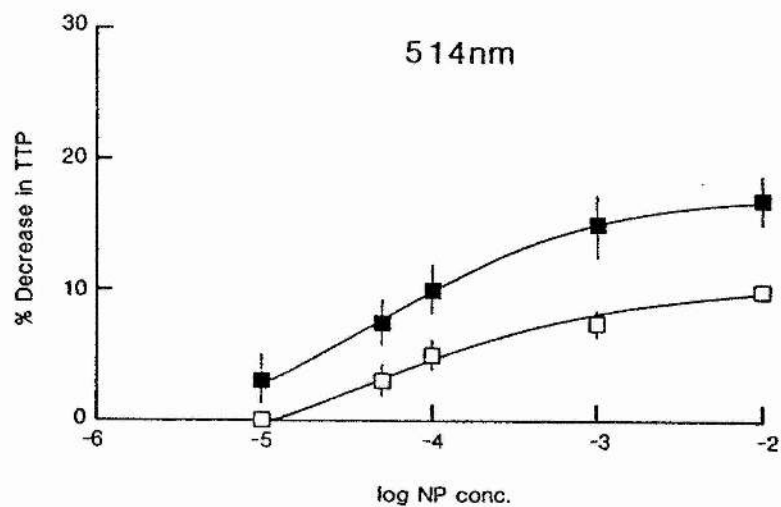
The percentage decrease in the maximum rate of rise of tension ( $dp/dt$ ) is plotted against  $\log [NP]$  for each illumination condition. Each point represents the mean (+ SE.) from 5 experiments with both 3mW (open symbols) and 20mW (closed symbols) being plotted on the same graph for each wavelength.



## Chapter 4

### Figure 4:11

The percentage decrease in the time to peak tension (TTP) is shown plotted against [NP] for each illumination condition. Each point represents the mean value (+ SE.) of 5 experiments. Both 3mW (open symbols) and 20mW (closed symbols) are plotted on the same graph for each wavelength.



two illumination conditions may have the same effect on  $dp/dt$  (i.e. 20mW 457nm & 20mW 488nm), the overall reduction in peak twitch tension can still be greater at the shorter wavelength due to a greater effect on the TTP.

The reduction in peak tension seen at lower [NP]'s, especially under less effective illuminations, is mainly due to a decrease in the rate of tension generation as the TTP is virtually unchanged. However, at higher [NP]'s and more effective illuminations, the effects on  $dp/dt$  are maximal and any further decrease in peak tension is due to a shortening of the TTP. Examples of these effects on the twitch shape can be seen in figure 4:5. Similar changes can be seen during the timecourse of a single response, in that initially, only the rate of tension generation is reduced, but as the concentration of active agent increases so the time to peak tension becomes shortened (see fig.4:7).

#### viii. Measurement of Aquapentacyanoferrate production following exposure of Nitroprusside to laser light.

The preceding results suggest that in order for NP to have any inotropic effect, it must first be exposed to light. The magnitude of the resulting effect is then dependent upon the quantity and wavelength of light used. Evidence in the literature suggests that NP is extremely photosensitive and readily undergoes photolysis to yield a number of photochemical products. It therefore seems likely that one or more of these is responsible for altering the twitch. We can therefore anticipate that the magnitude of the physiological

## Chapter 4

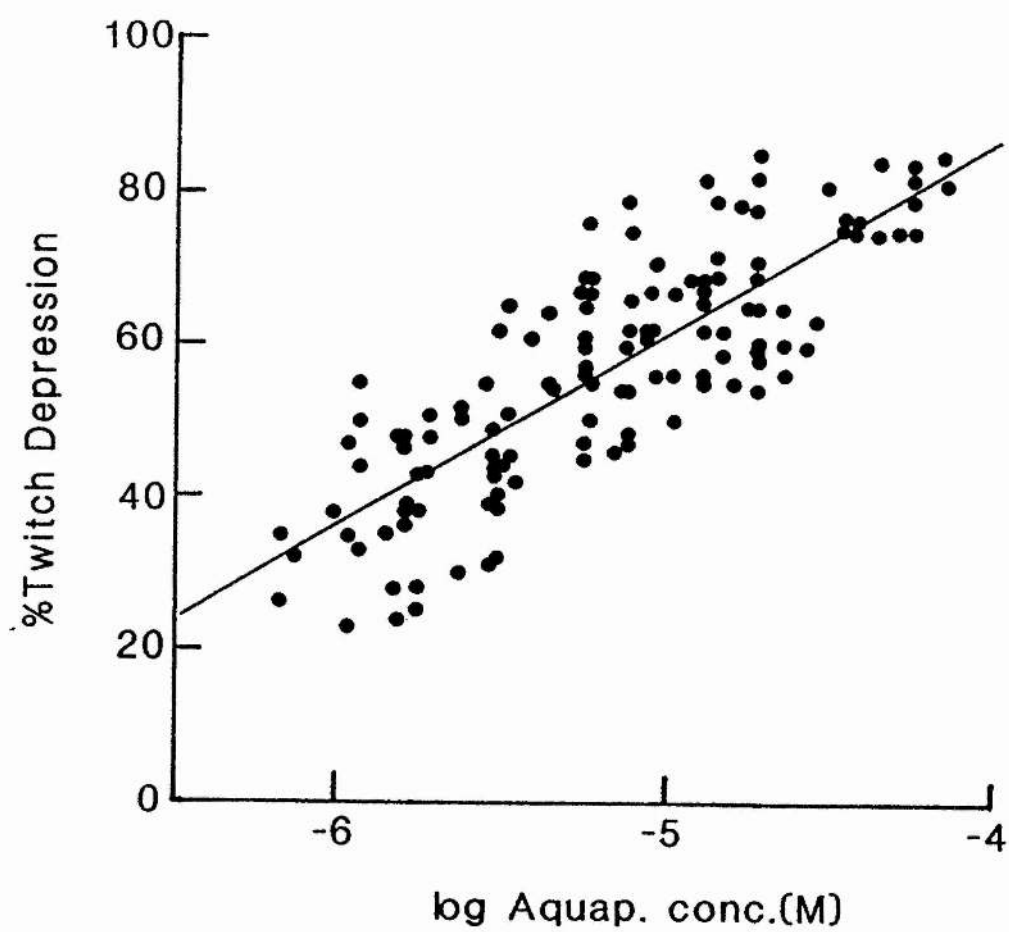
response is correlated with the efficacy of the photolytic process. This was investigated directly by monitoring the production of aquapentacyanoferrate by measuring its concentration in the chamber effluent. Measurements were made for the various illumination conditions over a range of NP concentrations. The results are shown in figure 4:12. As each individual measurement is plotted the scatter is large. There is however a high correlation ( $r^2=0.651$ ;  $p > 0.001$  for 124 degrees of freedom) between the depression of the twitch and the breakdown of NP. The calculated best fit line shows that a 50% depression of the twitch is associated with the photolysis of 3.7  $\mu\text{M}$  of NP.



## Chapter 4

**Figure 4:12**

The concentration of aquapentacyanoferrate was measured spectrophotometrically in the effluent of 130 experiments and plotted against the twitch depression in each case. The experiments involved a variety of different intensities, wavelengths and concentrations of NP. Linear regression analysis on the data revealed a high degree of correlation (  $r^2 = 0.651$  for 124 degrees of freedom:  $p > 0.001$ ) with a best fit line as plotted.



Discussion

The major conclusion to be drawn from these results is that the nitroprusside anion per se does not affect the twitch. The negative inotropic effect previously attributed to NP (Flitney, Moshiri & Singh, 1980) comes instead from one of its photolytic products. The extent of the inotropic action therefore depends on the amount of product. Although this is related to the initial [NP], it will also depend upon the efficiency of photolysis. The higher the intensity of illumination, then the more photons of light are available to react with the NP molecules such that more product is formed. In terms of the physiological responses, this manifests itself as a leftward and upward shift of the NP-dose response curve. The wavelength chosen also influences the photolytic process. As already mentioned in the introduction, particular wavelengths induce specific transitions in the electronic distribution of the molecule and therefore create different products. These wavelengths correspond with the peaks in the absorption spectrum. In the experiments described here, the relatively long wavelengths that are used (>457nm) are all associated with the photoreaction that liberates the nitrosyl moiety from the parent molecule (Wolfe & Swinehart, 1975). The absorption band most effective at cleaving this bond occurs at ca 400nm, and so wavelengths closer to this are more efficient at splitting NP (Wolfe & Swinehart, 1975). The order of efficacy in these experiments is therefore 457.9 > 488 > 514.5 nm. Illumination at 457nm would generate more of the physiologically active component than 488 or 514nm. This is

supported by the finding that it requires 20mW at 514nm to elicit the same response as only 3mW at 457nm. The leftward shift in the NP-dose response curves at constant intensity is also indicative of the greater efficacy of the shorter wavelengths.

The effects on the shape of the twitch presumably also depend on the concentration of the active agent formed during photolysis. This product seems to affect the mechanism of tension generation only at low concentrations, without changing the time to peak tension. Under conditions that favour its formation however, the effects on the TTP become more marked, indeed, the TTP can still be reduced when  $dp/dt$  is maximally depressed. It is interesting that the NP-dose response curves for the effects on these two parameters differ. The maximum rate of rise of tension varies sigmoidally with [NP], which has always the same maximal value of 55%, and is shifted to the left under more efficient illumination conditions. The relationship between [NP] and TTP is curvilinear with both the maximal value and slope being dependent on the illumination condition. This may mean that the physiologically active species depresses the twitch via two distinct mechanisms.

As discussed in the general introduction, NP is thought to act by increasing the intracellular concentration of cGMP (Flitney, Moshiri & Singh, 1980). In frog ventricular cells, most of the Ca required to activate the contractile machinery enters through the sarcolemma (Chapman, 1983). Cyclic GMP is reported to reduce the calcium influx during the action potential (Wahler & Sperelakis, 1985). A decrease in

the availability of Ca for the contractile apparatus would therefore reduce its tension generating ability. It is interesting to note that the NP-dose response curve for peak tension is influenced by the illumination conditions in a way that appears to result from a combination of the effects on both  $dp/dt$  and TTP.

Clearly, the results presented here pose an important question; what is the chemical identity of the physiologically active species formed during photolysis? According to Wolfe & Swinehart (1975), the conditions used in these experiments, namely air saturated buffered Ringer at pH 7.0 and relatively long wavelength irradiation, would favour production of aquapentacyanoferrate(III) (Aq) and nitric oxide (NO). NO is well known for its ability to stimulate guanylate cyclase directly and so must be the obvious candidate. Evidence in support of this hypothesis is presented in later chapters.

Meantime, we can note that the concentration of the active substance generated by photolysis would be related to the [Aq] formed. NO, for example, is produced with a 1:1 stoichiometry to [Aq]. Measurement of the [Aq] formed therefore gives an upper estimate of the concentration of the active species formed under any given set of experimental conditions. The data shown in figure 4:12 illustrates that the negative inotropic response is proportional to the [Aq] generated which is suggestive of a causal relationship. However, the [Aq] formed by [NP]'s  $< 10^{-4}M$  cannot be determined accurately by spectrophotometry and so the data necessarily covers a narrower range, heavily weighted towards the top end of the log[NP]-response curve. Even so, it is

## Chapter 4

clear that the effective concentrations of active species produced are very much less ( $>100$  fold) than the [NP] used. In order to explore this relationship further, a more complete dose response curve is required and a method for estimating the [Aq] generated at lower [NP]'s is described in the following chapter.

## CHAPTER 5

A procedure for estimating  
the extent of photolysis of nitroprusside.

Introduction

The previous chapter established that the physiological potency of 'nitroprusside' depends upon the extent to which it undergoes photolysis. The inference is that an active photolytic product is responsible for the inotropic activity. The amount of this substance produced, and hence the size of the response, clearly depends on both the [NP] and the illumination conditions. In order to more fully understand the physiological efficacy of this substance, it is essential to relate the size of the response to the amount of photolysed NP over the entire range of [NP] and illumination conditions studied. This can be done in part by measuring the production of aquapentacyanoferrate (Aq) in the bath effluent. Unfortunately, measurements can only be made at [Aq]'s  $> 1\mu\text{M}$  and at these concentrations the effects on twitch tension are considerable. Data can therefore only be obtained for the top end of the dose response curve. In order to construct a complete dose response curve, [Aq]'s lower than  $1\mu\text{M}$  must be estimated. This entails determining the efficiency of breakdown of NP at concentrations that produce measurable amounts of Aq, and then using it to calculate the amount produced at much lower [NP]'s. This procedure must take into account all the factors that influence the photolytic reaction, such that the effective concentration of active agent generated in the exposure tube can be reliably estimated.



I. Determination of the estimation procedure.Theory

The [Aq] formed in the exposure tube is estimated from the absorbance change at 395nm:

$$[Aq] = A/\epsilon \quad \text{eqn.(1)}$$

where A = measured change in absorbance

$\epsilon$  = molar extinction coefficient for Aq at 395nm (=743  $M^{-1}cm^{-1}$ )

Under 'ideal' conditions when the overall efficiency is 1.0, the maximum amount of Aq that can be formed is when all the NP is photolysed (ie.  $[Aq]=[NP]$ ). The overall efficiency (E) of the photolytic process is therefore given by :-

$$E=[Aq]/[NP] \quad \text{eqn.(2)}$$

or

$$[Aq]=E [NP]$$

Under the dynamic conditions pertaining in the present experiments, the maximum attainable [Aq] is related to the [NP] by:-

$$[Aq]= p\phi(q/m)[NP] \quad \text{eqn.(3)}$$

where

p = the probability that every photon will interact with a molecule of NP

$\phi$  = the quantal efficiency of the photolytic reaction (moles.einstein<sup>-1</sup>)

q = photon flux (photons . sec<sup>-1</sup>)

m = number of molecules of NP flowing through the exposure tube (sec<sup>-1</sup>)

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It is clear that  $p$  and  $(q/m)$  are inversely related. Thus,  $p$  will tend towards unity when  $q \ll m$ , and towards zero when  $m \ll q$ . Under ideal conditions of flow and illumination,  $p=1$  and so :-

$$[Aq] = \phi(q/m)[NP] \quad \text{eqn. (4)}$$

from which we have that:

$$\phi = [Aq]/(q/m)[NP] \quad \text{eqn. (5)}$$

The photon flux ( $q$ ) is given by:-

$$q = \lambda I/h\nu \quad \text{eqn. (6)}$$

where  $\lambda$  = wavelength of light (m)

$I$  = intensity of illumination (W)

$h$  = Planck's constant ( $6.63 \times 10^{-34} \text{ J} \cdot \text{sec}^{-1}$ )

$\nu$  = velocity of light ( $2.99 \times 10^8 \text{ m} \cdot \text{sec}^{-1}$ )

and the number of molecules flowing through the tube per second by:-

$$m = [NP] \cdot F \cdot Av \quad \text{eqn. (7)}$$

where  $F$  = flow rate ( $\text{l} \cdot \text{sec}^{-1}$ )

$Av$  = Avagadro's number ( $6.02 \times 10^{23}$ )

Eqn. (5) has the dimensions of molecules.photon<sup>-1</sup> which is more usually expressed as moles.einstein<sup>-1</sup> (where 1 einstein =  $6.02 \times 10^{23}$  quanta). It is important to emphasise that eqn. (5) is only valid for ideal conditions of flow and illumination, when  $p=1.0$ . In practice, if  $q \gg m$ , caused either by increasing the intensity of illumination or by reducing the  $[NP]$ , or both, then the apparent value of  $\phi$ , which we denote by  $\phi'$  is given by:

$$\phi' = p \cdot \phi \quad \text{eqn. (8)}$$

Clearly, estimates of  $[Aq]$  generated in experiments using  $[NP] < 10^{-4}$ , where it is not possible to measure the

change in absorbance directly, can only be made when the relationship between  $p$  and  $q/m$  is known. This can be found for any given wavelength providing  $\phi$  is first determined under ideal conditions, since from eqn.(4) we see that:

$$p = [Aq]/(q/m)\phi[NP] = 1.0 \quad \text{eqn. (9)}$$

### Experimental results

#### 1. Effect of varying flow rate on Aq production

Eqn.(3) shows that  $[Aq]$  formed is directly proportional to the intensity of illumination ( $q$ ) and inversely proportional to the flow rate ( $m$ ). The importance of flow rate is shown in figure 5:1. Here, 10mM NP was exposed to 20mW light at 457nm. The flow rate was varied from 2.5 to 15 mls.min<sup>-1</sup>. Curve A shows that  $[Aq]$  decreases with increasing flow rate, from around 700uM at 2.5 mls.min<sup>-1</sup> to 100uM at 15 mls.min<sup>-1</sup>. Conversely curve B shows that the  $[Aq]$  is directly proportional to the transit time. The apparent value for the quantal efficiency ( $\phi' = p\phi$ ) is shown by the open squares (curve C) measured at each of the flow rates. The mean value is found to be 0.074. The consistency of these estimates of  $\phi'$  show that the method is able to compensate for large variations in flow rate, much greater than those encountered in the actual experiments.

#### 2. Relationship between $[Aq]$ formed and $q/m$

Eqn.(8) shows that  $[Aq]$  generated is linearly related to the product  $q/m$  and  $[NP]$  with a gradient of  $p\phi$ :

$$p\phi = [Aq]/(q/m)[NP] \quad \text{eqn. (10)}$$

Figure 5:2(A) shows the results of an experiment in which the production of Aq was measured over a wide range of

Figure 5:1

This figure shows a composite plot of the effects of varying the flow rate through the exposure tube, upon the photolysis of a 10mM NP solution exposed to 20mW 457nm laser light.

A - Relationship between the concentration of aquapentacyanoferrate (Aq) produced under different flow rates (F).

B - Relationship between the Aq concentration against the transit time (T) that each molecule spends in the exposure tube.

C - Plot of the apparent quantal efficiency  $\phi'$  (=  $p\phi$ ) as calculated from eqn.10, for each flow rate value. The consistency of this value suggests that the equation can compensate for large variations in flow rates. The line shows the mean  $p\phi$  value, which is 0.074.

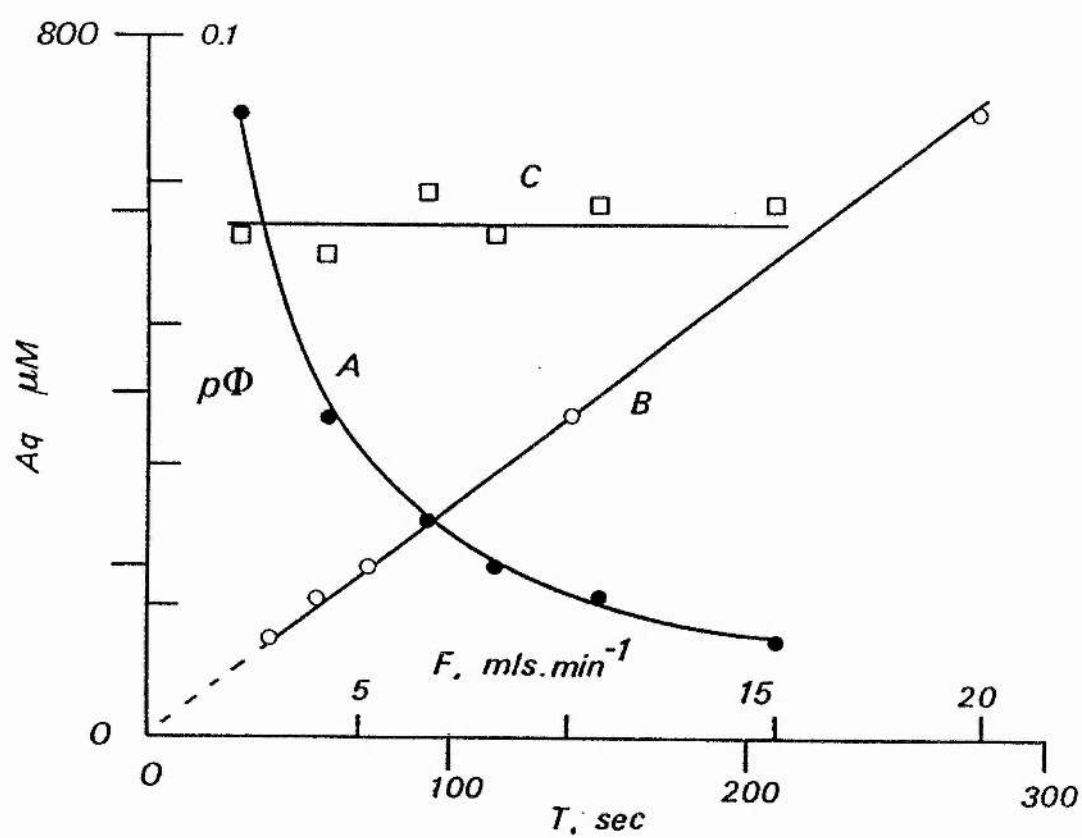
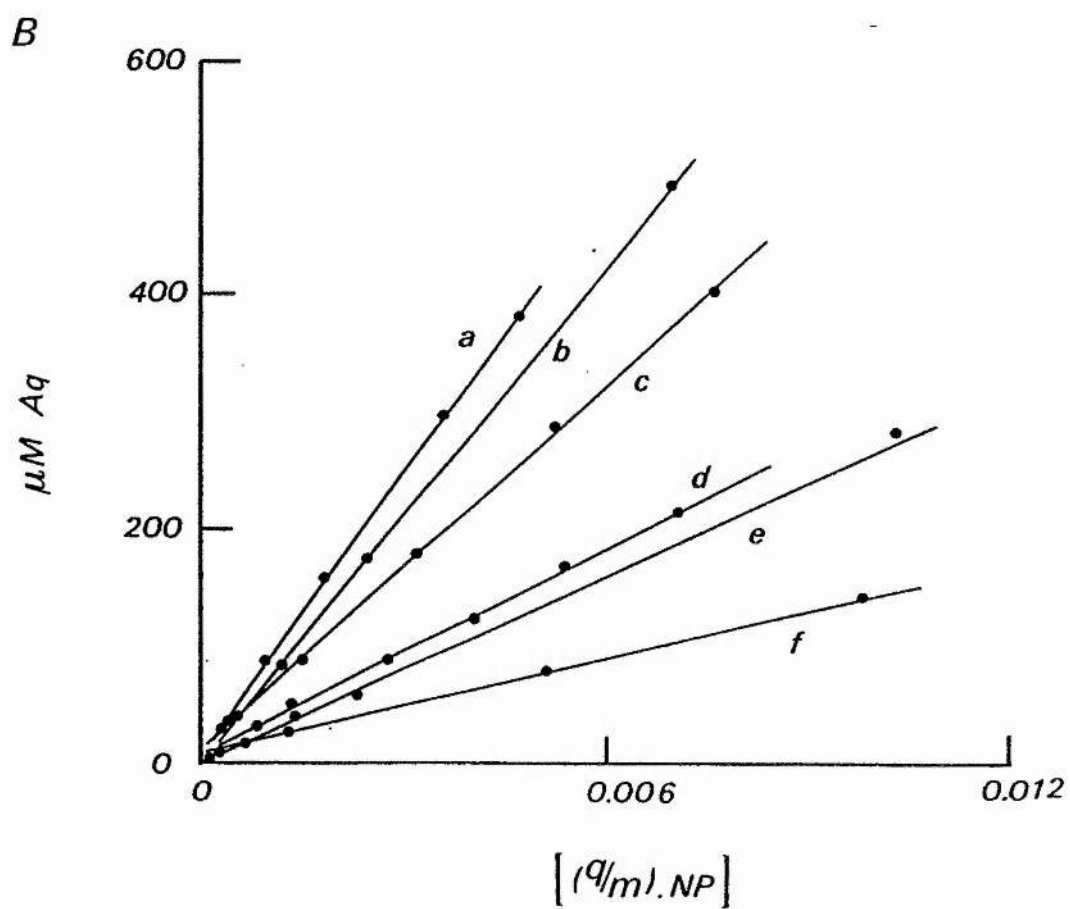
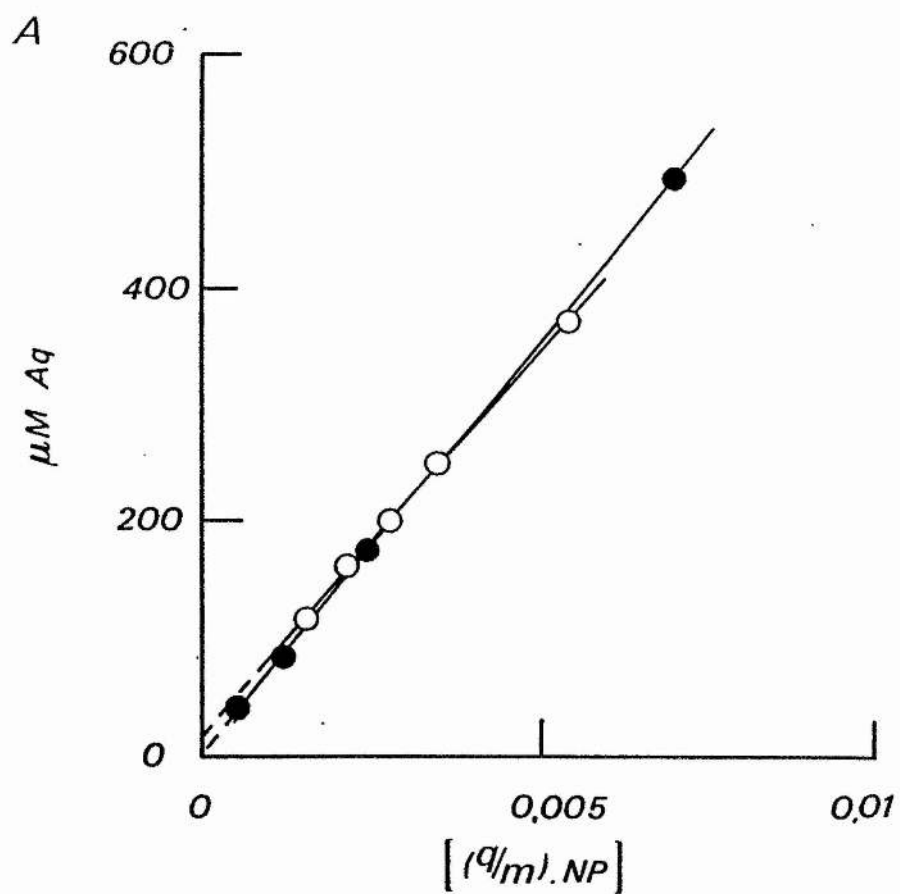


Figure 5:2

A - Graph of the linear relation between  $A_q$  produced and  $(q/m).NP$  for a single experiment in which either the flow rate (open dots) or intensity of illumination (closed dots) was varied during an exposure of 10mM NP perfusate by 457nm laser light. The single linear relation implies that the  $(q/m).NP$  factor compensates for both changes in flow rate or intensity.

B - The linear relation between  $A_q$  and  $(q/m).NP$  under conditions similar to A, are plotted for different wavelengths of incident light according to the key below. Although the linear relationship is constant for variations in intensity, the gradient is dependent on the wavelength of illumination.

a	454 nm
b	457 nm
c	488 nm
d	496 nm
e	514 nm
f	528 nm



$q/m$  values. This was achieved by altering  $q$  and  $m$  independently i.e. either by varying flow rate while keeping the intensity constant (open circles), or by changing the intensity while keeping flow rate constant (closed circles). The data are almost superimposable and yield the same linear relationship between  $[Aq]$  and the product  $(q/m)[NP]$ . Thus, evaluating  $q/m$  is able to compensate for differences in the efficiency of photolysis which result from variations in the flow rate and/or intensity of illumination.

Experiments using a different  $[NP]$  and different wavelengths (see legend fig.5:2(B)) yield a similar linear relationship between  $[Aq]$  and  $(q/m)[NP]$ . Note, however, that the gradient of the lines are different and that they vary inversely with wavelength.

### 3. Effect of varying $[NP]$ on $p$

The probability  $p$  that each photon will interact with a molecule of NP is determined by several factors. First, it depends upon the geometry of the exposure tube, in as much as this affects the dispersion of light within the lumen. This is an invariant property unique to the tube being used. Secondly,  $p$  will depend on  $[NP]$  flowing through the tube. The mean free distance between molecules will vary inversely as the cubed root of the  $[NP]$ . Thus, for a 1M solution the mean free path is estimated to be 1.18nm, increasing by a factor of 100 times to 118nm on diluting the solution to  $10^{-6}M$ . Clearly, this is an important determinant of  $p$  since photons which pass through the tube without encountering a molecule of NP are ineffective.

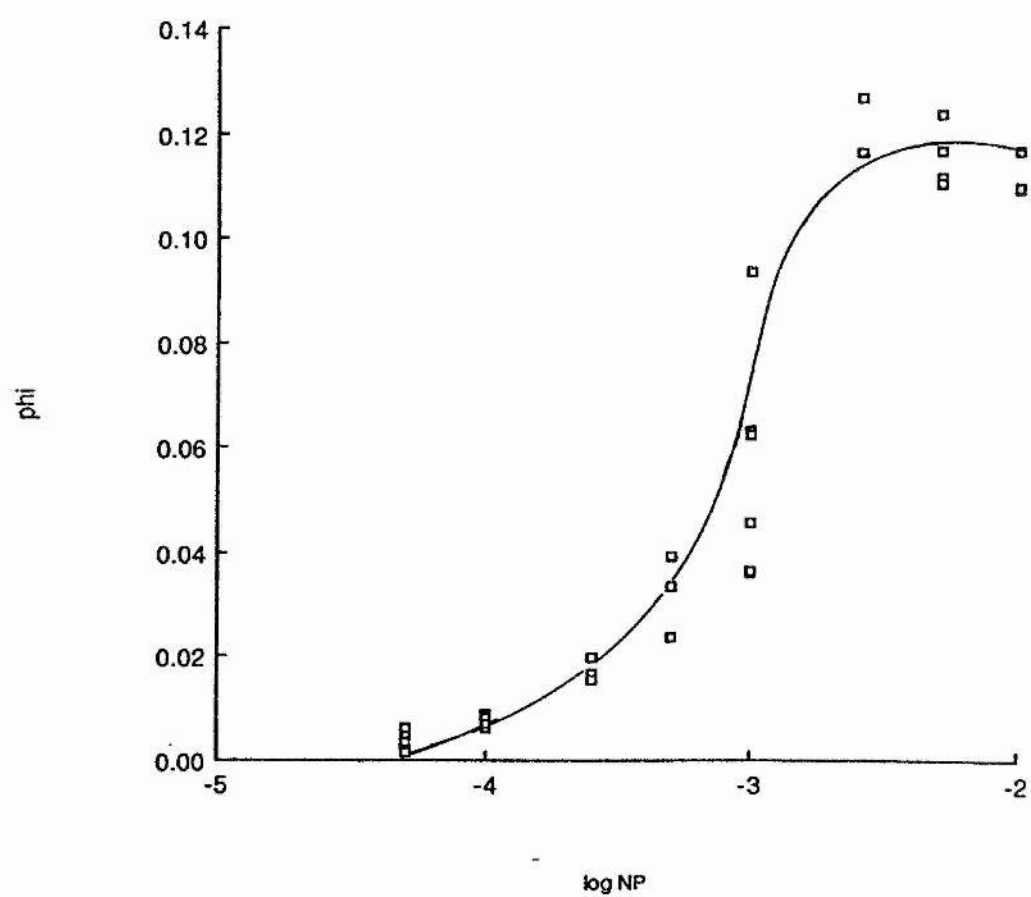
The real value for the quantal efficiency of the



## Chapter 5

### Figure 5:3

The value of  $p$  was calculated (via eqn. 9) for a range of NP concentrations under constant flow (10mls/min.) and illumination conditions (20mW 457nm ). The value increases with increasing NP conc. until it reaches a maximum plateau value above  $2.5 \times 10^{-3}$  M NP. Above this concentration, further increases in NP do not increase  $p$  such that the photolytic conditions are optimal for these experimental conditions. If we assume that  $p=1.0$  under these conditions, then the plateau value of  $p$  is equivalent to the quantal efficiency for the wavelength involved. In this experiment this is estimated to be  $0.1152 (\pm 0.0021)$  mol/einstein for 457nm ( $n=8$ ).



photolytic process can be found from experiments such as that illustrated in figure 5:3. Here,  $\phi'$  ( $=p\phi$ ) was measured over a range of [NP]'s under conditions of constant flow ( $10\text{mls.min}^{-1}$ ) and illumination ( $20\text{mW}$ ,  $457.9\text{nm}$ ). These results show that  $\phi'$  increases with increasing [NP] until it reaches a limiting value above  $2.5 \times 10^{-3}\text{M}$ . Further increases in [NP] has no further effect on  $\phi'$ . Thus, at  $[\text{NP}] > 2.5\text{ mM}$ ,  $p$  is maximal ( $=1$ ) and  $\phi' = \phi$ . This data returned a value for  $\phi$  at  $457.9\text{nm}$  of  $0.1151 \pm 0.0021\text{ moles.einstein}^{-1}$  ( $n=8$ ). Similar experiments for  $488\text{nm}$  and  $514\text{nm}$  wavelengths gave values of  $0.053$  and  $0.023\text{ mole.einstein}^{-1}$  respectively.

Figure 5:4 shows these estimates of  $\phi$  plotted as a function of wavelength together with two additional values at  $366$  and  $436\text{nm}$  taken from Wolfe and Swinehart's (1975) measurements. The absorption spectrum for exposed NP ( $5\text{mM}$ ) is also illustrated and the data have been scaled at  $366\text{nm}$  so that they superimpose. The estimates of  $\phi$  increase with decreasing wavelength by a factor of  $15.9$  over the entire range. The peak in the absorption spectrum (at  $395\text{nm}$ ) is the optimal wavelength for cleaving the nitrosyl ligand. The value of  $\phi$  should therefore be maximal at  $395\text{nm}$ , but this wavelength was not available by the laser used in these experiments.

#### 4. Relationship between $p$ and $q/m$

In order to estimate the  $[\text{Aq}]$  formed at low [NP]'s the relationship between  $p$  and  $q/m$  must be found. Since  $\phi$  was estimated under ideal conditions as described above,  $p$  can be obtained from eqn.(9) under any set of experimental conditions.

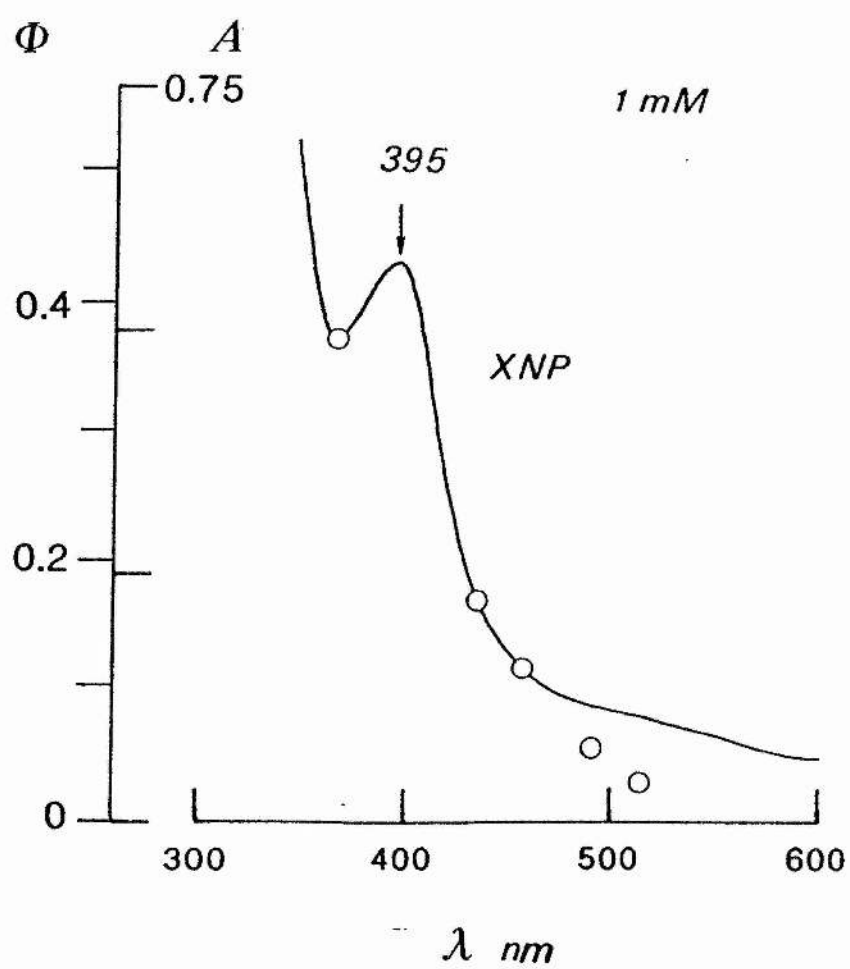
$$p = [\text{Aq}]/(q/m)\phi[\text{NP}]$$

## Chapter 5

Figure 5:4

Quantal efficiency values, estimated for each of the wavelengths used in this study, are shown plotted on the scanning absorption spectra of photolysed NP along with other values from the literature. This illustrates the wavelength dependence of the photolytic reaction, and why the quantal efficiency values increase with shorter wavelengths. The quantal efficiency values are given below;

nm	mole/einstein
366	0.37 (Wolfe & Swinehart, 1975)
436	0.18 ( " " " " )
457	0.1152
488	0.0530
514	0.0233



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Fig.5:5 shows the relationship between  $\log p$  and  $\log (q/m)$ , for a range of flow rates, intensities of illumination, wavelengths and [NP]'s. The value of  $\log p$  is seen to increase linearly from around  $-1.5$  at  $(q/m) = 10$  to reach a plateau value of around  $0$  at  $q/m < 0.1$ . Linear regression analysis shows that:

$$\log p = -0.695 + (-0.788(\log (q/m))) \quad \text{eqn. (11)}$$

(regression coefficient = 0.99)

This relationship can be used to estimate  $\phi$  from the data presented earlier, in fig.5:1, curve C. At a flow rate of  $10 \text{ mls. min}^{-1}$ ,  $p$  is estimated to be  $0.64$ , which since the value of  $\phi'$  is  $0.074$ , gives a corrected value of  $\phi = 0.1156$  ( $0.074 \times 0.64$ ). This closely compares with the  $\phi$  value for this wavelength ( $=0.1152$ ) estimated under optimal experimental conditions (ie. the plateau values in fig.5:3).

The predictive value of the method can be tested by comparing theoretical values for [Aq] generated under a given set of conditions with those actually measured. This is illustrated by the data in fig.5:6. The relationship is linear with a slope of  $1.02$  and a least squares regression coefficient of  $0.99$ . The data include measurements at all three wavelengths, for different flow rates, [NP]'s and illumination intensities.

In the analysis that follows, the assumption is made that the relationship between  $p$  and  $q/m$  also holds true at very low [NP]'s, and that the [Aq] can therefore be reliably estimated by extrapolation of the linear portion of fig.5:5. It is probable that this assumption is justified because the fixed geometry of the tube on one hand and the physical

Figure 5:5

The probability factor  $p$  was calculated for a range of flow rates, intensities, wavelengths and [NP]s. Log  $p$  when plotted against  $\log (q/m)$  linearized the relationship ( $R^2=0.99$ ), up to a maximum plateau value when  $p=1.0$ . This means that ideal photolytic conditions (ie. when  $p=1.0$ ) exist when  $q/n$  is small, and that at higher values of  $q/n$  ( $> 0.128$ ), the value of  $p$  decreases with the relationship;

$$p=10^{(-0.695 + (-0.788(\log q/m)))}$$

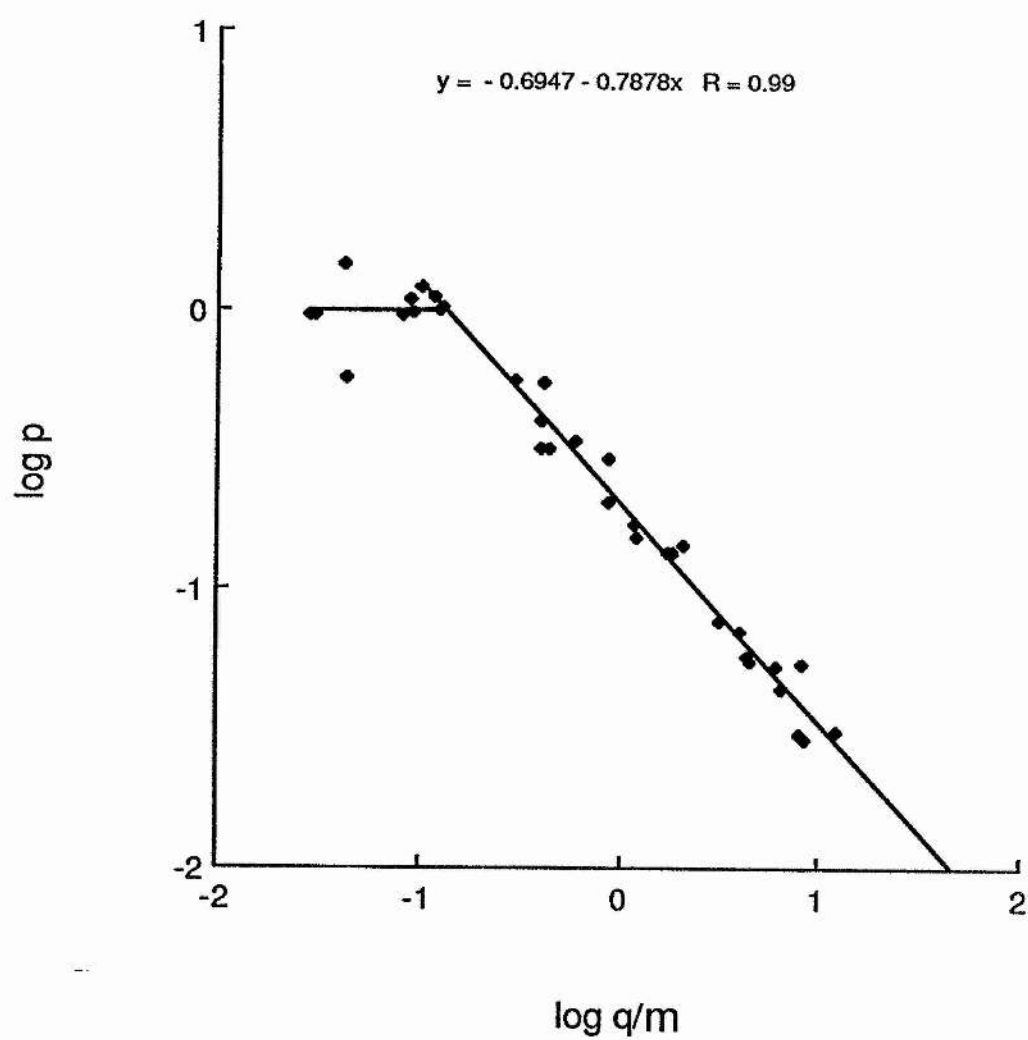
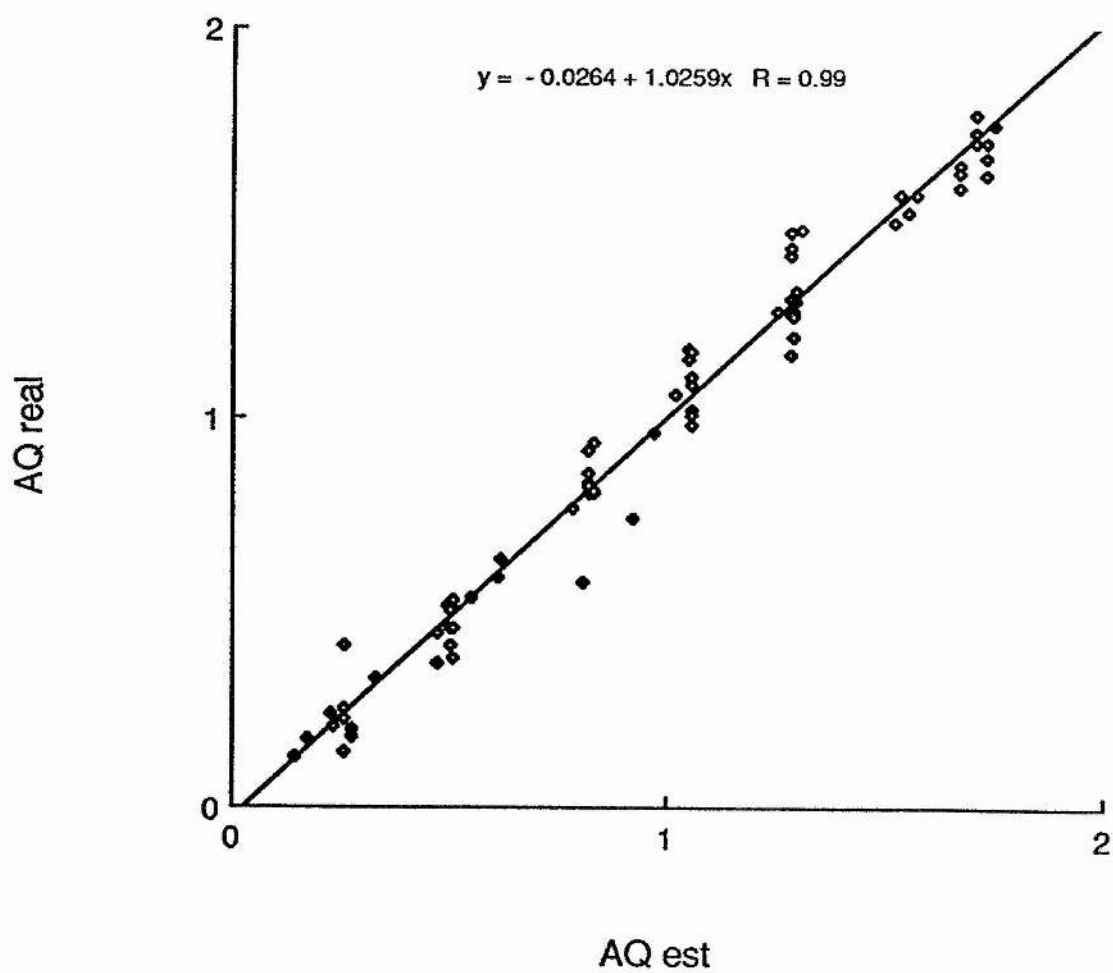




Figure 5:6

The estimation procedure was tested against a wide range of experimental conditions in which the Aq concentration was directly measured. The estimated value was plotted against the real measured value as shown. There is a very good linear correlation ( $R^2 = 0.99$ ) between the two sets of results, with a slope of nearly 1.0 which suggests that the estimation procedure is accurate.



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relationship between intermolecular distance and  $[NP]$  on the other are the major determinants of  $p$ . At the very least, this approach provides an order of magnitude estimate of the degree of photolysis which can then be related to the response shown by the muscle.

## II. Relationship between degree of photolysis and physiological responses

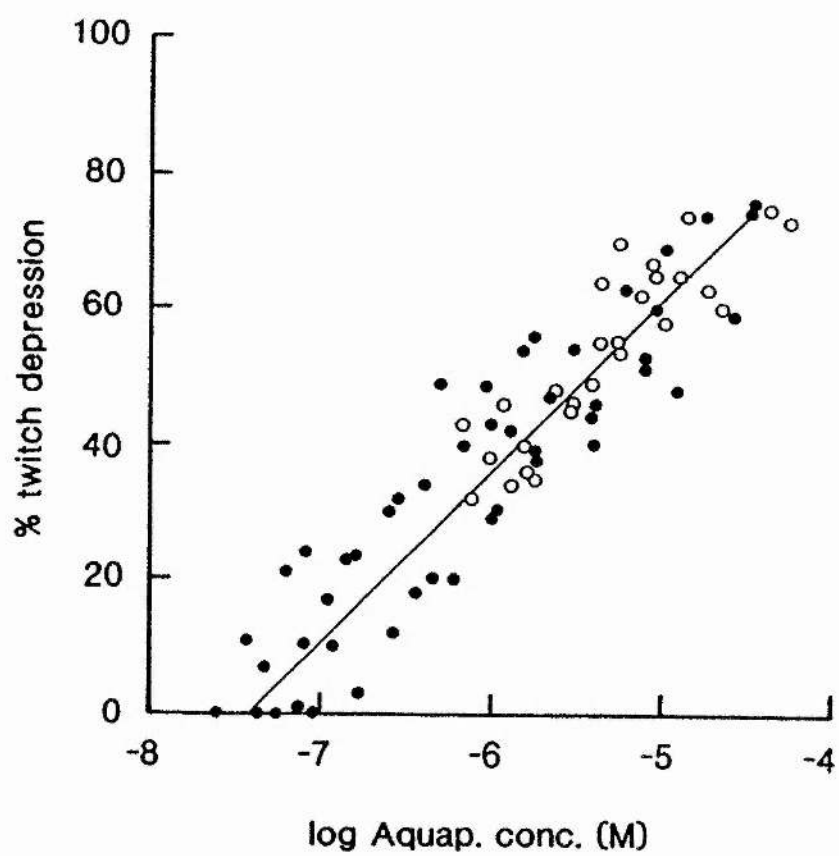
The several NP dose response curves shown in the previous chapter, obtained using different wavelengths and intensities of illumination, are all transformed into single curves by plotting the data as a function of the [Aq] formed in the exposure tube. Figure 5:7 shows the relationship between  $\log[Aq]$  and peak twitch tension, using data previously included in the individual dose-response curves of figure 4:8. Figure 5:8 shows the relationship for  $dP/dt$  (A) and TTP (B) plotted against  $\log[Aq]$ , the data for which was previously shown in figs.4:10 & 4:11 respectively. The experimentally measured [Aq], indicated by the open symbols, are shown together with those estimated using eqn.(3) (closed symbols). Each data point represents averaged responses and [Aq]'s, as determined for a standardised set of experimental conditions (flow rate, wavelength, intensity of illumination and [NP]). The [Aq] generated in the exposure tube is here used as an indicator of the photolytic reaction. Stoichiometrically, one molecule of NP generates a single molecule of Aq, so the abscissa for each curve can be directly related to NP breakdown.

There are three points to emphasise concerning these results;

First, by taking account of all the experimental variables, and using these to estimate the quantity of NP photolysed, makes it possible to translate the data points onto single log dose response curves. Significantly, those points for which [Aq] was measured directly exactly

Figure 5:7

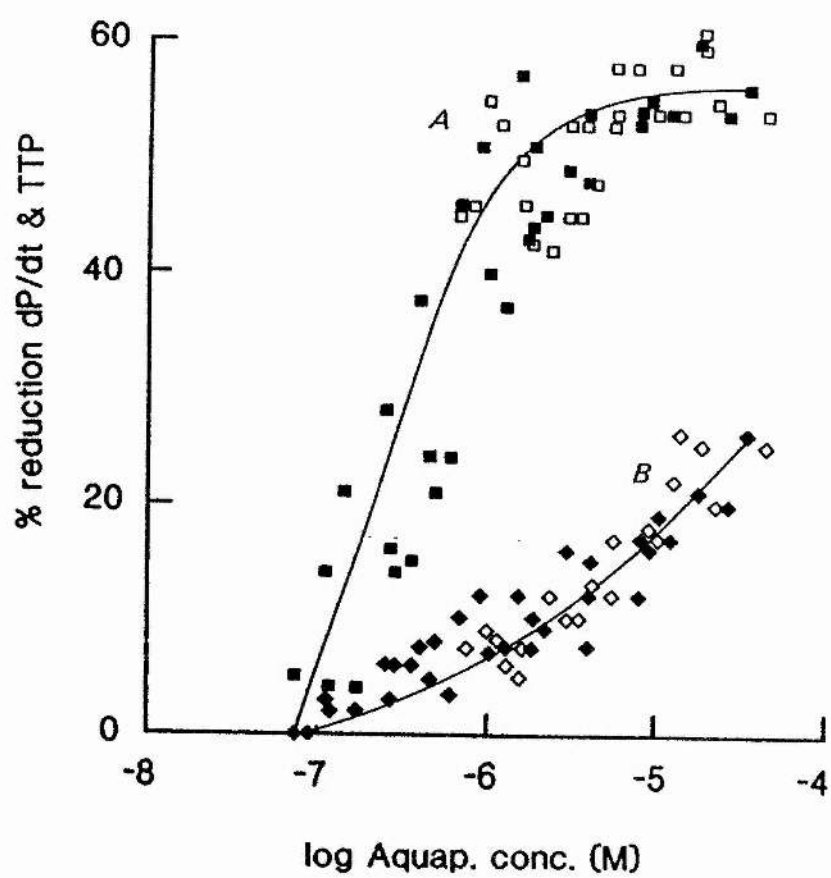
The log dose response curves to photodegraded NP, as determined by the estimated Aq concentration, are shown. All three wavelengths and two intensities (closed circles) lie on the same dose response curve which is almost linearly related to twitch depression. Measured Aq values (open circles) are also lie on this curve, which lends further evidence that the estimation procedure is accurate.



## Chapter 5

### Figure 5:8

The percent reduction in both the maximum rate of rise of tension ( $dP/dt$ ;  $\square$ ) and time to peak tension (TTP;  $\diamond$ ), are plotted against  $\log[A_q]$ . Both estimated (closed symbols) and measured (open symbols)  $A_q$  values are shown, incorporating a variety of wavelengths, intensities and  $[NP]$ s. The  $ED_{50}$  values for  $dP/dt$  is  $3.9 \times 10^{-7}$  whereas it is  $4.2 \times 10^{-6}$  for TTP.





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superimpose on the upper end of each curve.

Secondly, physiological effects are detectable when the experimental conditions are such that only 50-100nm NP is photolysed. The low threshold concentration is an indicator of the extreme sensitivity of the preparation to the active agent formed.

Third, the substance generated by photolysis, affects the three twitch parameters differently. The  $ED_{50}$  values increase in the order 0.4 $\mu$ M (dP/dt), 1 $\mu$ M (peak twitch) and 4.2 $\mu$ M (TTP). The curve relating dP/dt to [Aq] is particularly steep rising to a maximum plateau over one decade from around  $10^{-7}$  to  $10^{-6}$  M. In comparison, those which relate peak twitch and TTP to [Aq] both extend over approx. 2.5 decades and show no tendency to level off even when [Aq] reaches 40-50 $\mu$ M.

## CHAPTER 6

Experiments to identify the physiologically  
active photolytic product.

Introduction

The results presented thus far have established that a photolytic product of NP, produced in nM- $\mu$ M amounts, is responsible for its physiological effects. The experiments now to be described were undertaken in an attempt to identify the substance concerned. As noted earlier, the products of photolysis will depend upon the ambient conditions of exposure, solvent used, pH, etc, and for the conditions used throughout this study the reaction sequence would be expected to follow Wolfe and Swinehart's (1975) scheme (see fig.6:1). Here, the primary photochemical event splits NP into pentacyanoferrate and nitric oxide (NO). The pentacyanoferrate is then rapidly hydrated to form aquapentacyanoferrate (Aq). NO does not react directly with water, but it readily combines with dissolved oxygen to form dinitrogen tetroxide or nitrogen dioxide, which dissociates in water to produce nitrous acid and nitrate. Nitrous acid itself dissociates to produce nitrite, nitrate, NO and hydrogen ions (Wolfe & Swinehart, 1975). There are thus a number of substances that must be considered potential candidates for the physiologically active agent.

Inspection of fig.6:1 shows that the fate of NO formed during photolysis is  $O_2$  dependent whereas that of Aq is not. This means that the equilibrium between the several products can be perturbed in a predictable manner by changing the  $O_2$  tension: raising it will favour the production of  $NO_2$  and  $NO_3$  at the expense of NO, while reducing it will suppress this



## Chapter 6

pathway such that more NO remains in solution. Notice that neither of these interventions will alter the fate of Aq.

There is another related consequence of changing  $[O_2]$  which is relevant in the present context. The production of superoxide anions ( $O_2^-$ ), which are highly reactive and combine readily with NO to form  $NO_3^-$ , will be favoured at high  $O_2$  tensions. Superoxide ions can be generated by the reduction of dissolved  $O_2$  using ferrous ( $Fe^{2+}$ ) ions. Conversely, cupric ( $Cu^{2+}$ ) ions, which have a redox potential lower than that of  $O_2$ , will inhibit its formation. The enzyme superoxide dismutase (SOD), which catalyses the conversion of  $O_2^-$  to hydrogen peroxide, can also be used as a means of assessing the importance of superoxide ions (and by inference that of NO) in determining the inotropic response.

Finally, the haem group of haemoglobin (Hb) has a strong affinity for NO and can therefore be used as a NO scavenger. The reaction is extremely rapid (Antonini & Brunori, 1971) and the product formed, nitrosyl haemoglobin (Hb-NO), is relatively stable. Its formation can be detected by conventional spectrophotometry and because of the unpaired electron associated with NO, it also generates a characteristic electron paramagnetic resonance (EPR) signal.

These considerations form the basis for the series of experiments described in this section. It will be seen that the results support the hypothesis that the inotropic response is due to NO.

Results

## Effects of varying oxygen tension on responses to photolysed NP

NP solutions with three different oxygen tensions were prepared from Ringers' solution saturated with either air, pure oxygen or pure nitrogen. The solutions were continuously bubbled throughout the experiment to ensure that the  $[O_2]$  remained constant. This was regularly checked using an oxygen electrode. The  $[O_2]$ 's obtained were  $74 \pm 0.020$   $\mu M$  ( $N_2$  saturated),  $260 \pm 0.014$   $\mu M$  (air saturated) and  $910 \pm 0.080$   $\mu M$  ( $O_2$  saturated).

The experimental protocol first established the 'normal' response to photolysed NP (20mW, 457.9nm) in air saturated solutions. When peak twitch tension had reached its new steady state level, the perfusate was switched to  $O_2$  saturated solution containing the same [NP]. Again the twitch was allowed to stabilise before switching the perfusate over to  $N_2$  saturated NP solution. The preparation was allowed to recover in air saturated (normal) Ringers' solution before repeating this procedure with a different [NP]. Control experiments showed that changing the oxygen tension alone, in either Ringers' solution or protected NP solution, had no effect on the twitch. Moreover, the efficiency of photolysis was found to be independent of oxygen tension as demonstrated by the [Aq] measurements listed in table 6:1.

Figure 6:2 (A,B) shows recordings of responses to two [NP] at each of the three oxygen tensions. In panel A ([NP]=50 $\mu M$ ) the twitch depression in air saturated solution is slightly reversed by switching to oxygen saturated solution.

Table 6:1

[AQUAPENTACYANOFERRATE] <sup>*</sup>			
(μM)			
[NP]	N <sub>2</sub>	AIR	O <sub>2</sub>
10 <sup>-4</sup> M	2.83 ± 3.2	3.03 ± 0.19	3.10 ± 0.36
2.5 x 10 <sup>-4</sup> M	6.53 ± 0.12	7.53 ± 0.77	7.00 ± 0.84
5 x 10 <sup>-4</sup> M	12.10 ± 1.9	12.8 ± 1.82	12.4 ± 1.92
10 <sup>-3</sup> M	20.6 ± 3.8	18.9 ± 4.46	21.2 ± 4.07
5 x 10 <sup>-3</sup> M	51.1 ± 14.2	56.3 ± 10.4	58.9 ± 14.2
[O <sub>2</sub> ] (mmole.l <sup>-1</sup> ):	0.074 ± 0.02	0.26 ± 0.014	0.92 ± 0.08

STEADY-STATE CONCS.

FLOW RATE: 10 mls. min<sup>-1</sup>

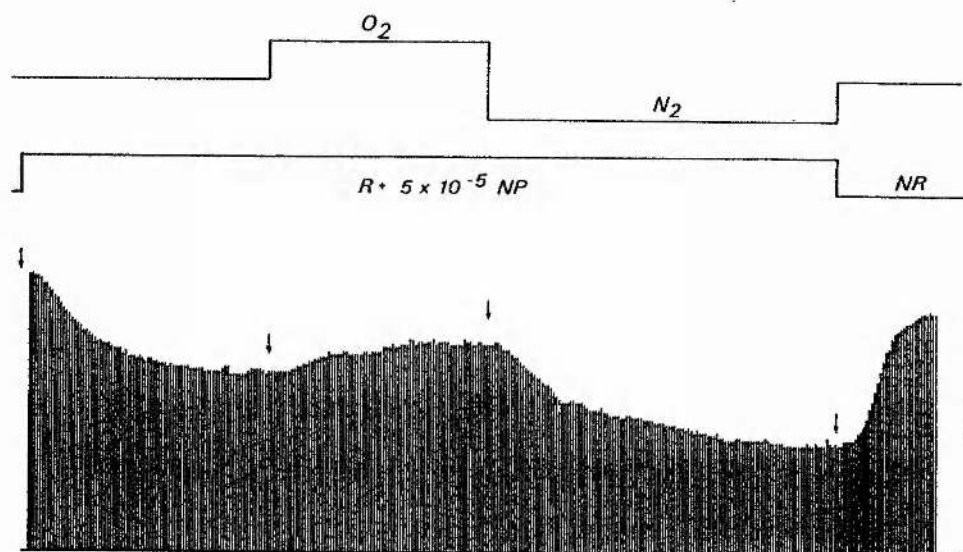
ILLUMINATION: 20 mW AT 457.9 nm.

Figure 6:2

The top bars in these panels depict the oxygen content of the perfusate, an upward deflection representing an increase, ( $O_2$ - oxygen saturated) whereas a downward deflection represents a decrease ( $N_2$ - nitrogen saturated) from the normal air saturated solution. The middle bar indicates the changeover of the perfusate from normal ringer to ringer containing either 50uM [NP] (panel A) or 1mM [NP] (panel B) which was constantly illuminated in the exposure tube by 20mW 457nm laser light. The computerised traces show the peak tension of every twitch, according to the scale bars on the right (each bar represents 0.05mN).

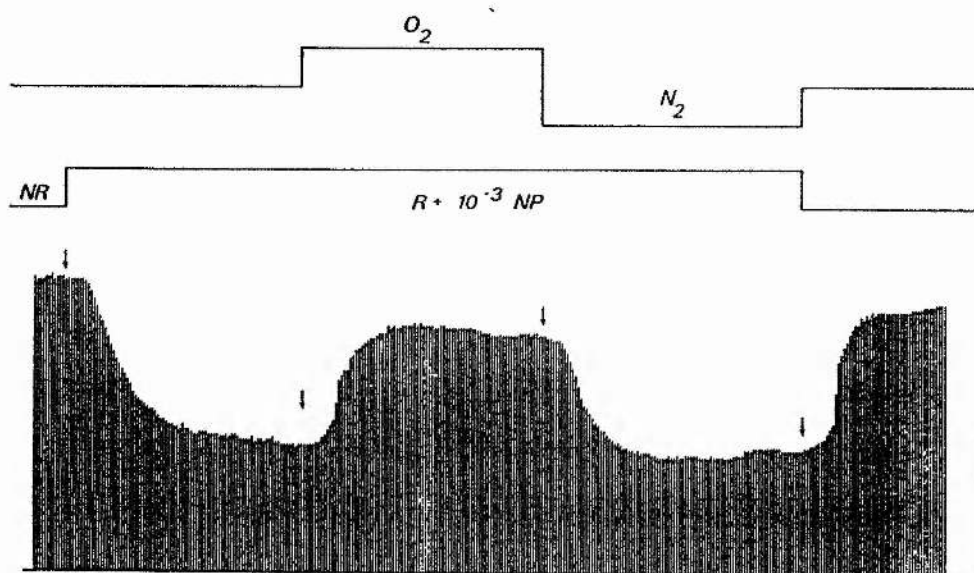


A



2 min.

B



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On changing to the low oxygen ( $N_2$  saturated) solution the reduction in twitch tension is significantly greater than that produced by the air saturated solution. Qualitatively similar changes are seen at the higher [NP] (=1mM) shown in panel B, although the reversal in high oxygen containing solutions is much greater.

The effects of varying the oxygen tension on the shape of the twitch is shown in fig.6:3 for three different [NP]'s and in fig.6:4 at different times during a response ([NP]=0.5mM). As found previously, the decrease in peak twitch tension is associated with reductions of both  $dp/dt$  and TTP tension. Once again, the extent to which these are affected is related to the overall reduction in peak twitch tension: small depressions are associated only with a decrease in  $dp/dt$ , whereas larger depressions are also associated with a shortening of the TTP. Significantly, the effects of varying the oxygen tension for a given [NP] closely mimic those produced by changing the [NP] at a fixed oxygen tension. Thus, enhanced responses in low oxygen tensions are associated with reductions in both  $dp/dt$  and TTP, whereas at high oxygen tensions the predominant effect is on  $dp/dt$  and TTP is barely affected. These results show that either altering the [NP] at a fixed oxygen tension, or varying the oxygen tension for a fixed [NP], affect the twitch in qualitatively similar ways. The inference to be drawn is that they serve the same purpose, in affecting the concentration of the physiologically active agent formed during photolysis. Figure 6:5 shows the accumulated data from a series of experiments in which responses were recorded for [NP]'s spanning the range  $5 \times 10^{-7}$

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### Figure 6:3

Individual steady state twitches of three different NP concentrations of at various oxygen tensions, are shown superimposed on the preceding control twitch. The differentiated form of these traces are drawn underneath on the same time scale, to more clearly show the changes in twitch parameters. The scale bars on the right are 0.05mN for the twitch tension traces and 0.5 mN/sec for the differentiated traces, the time bar common to both is 0.5sec.

5uM NP

0.1mM NP

10mM NP

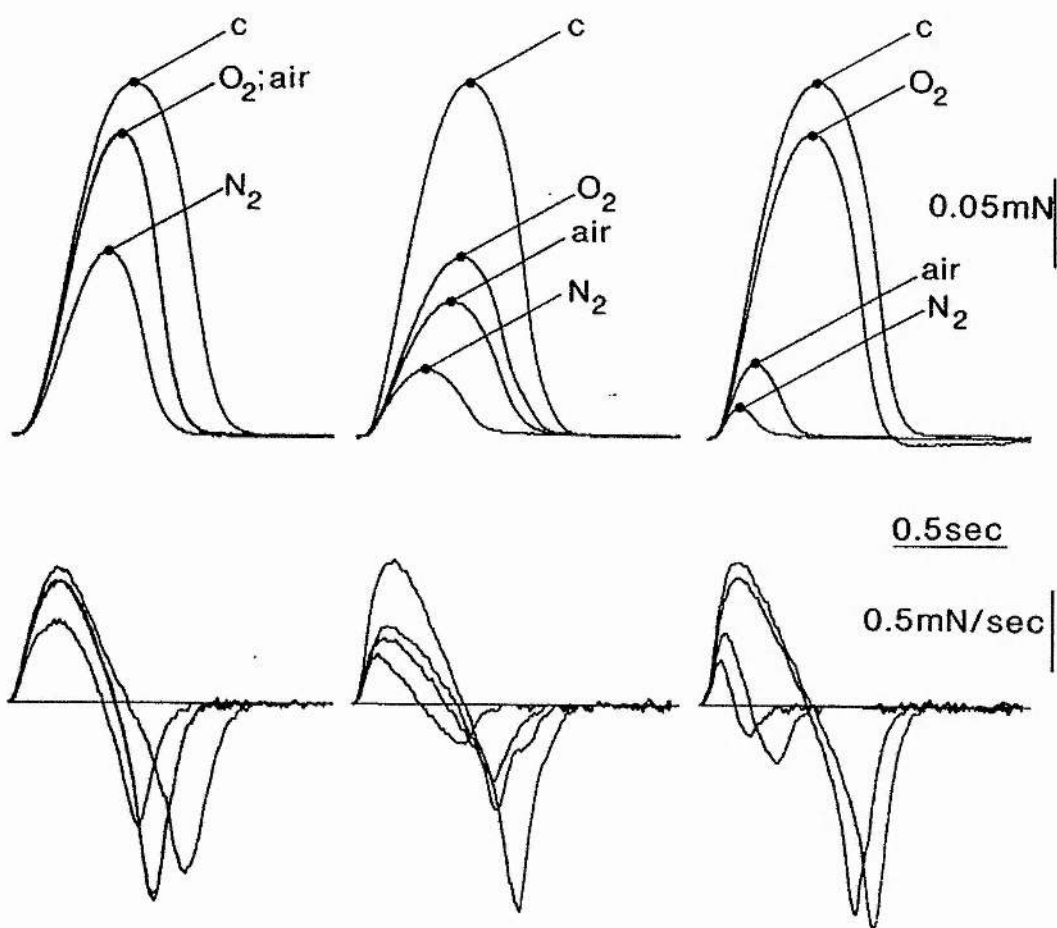
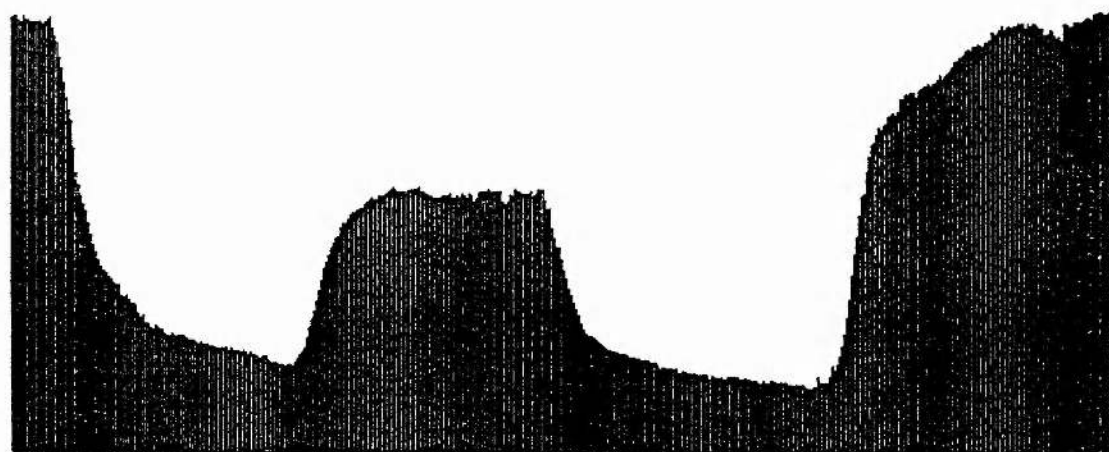
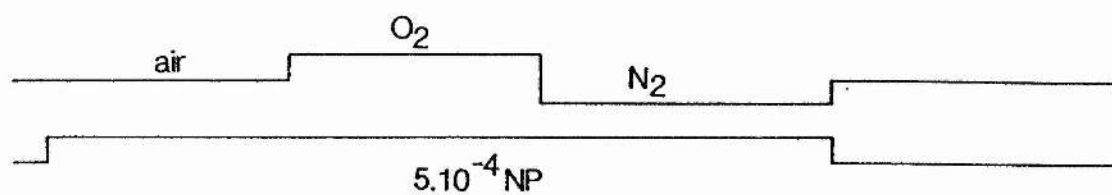


Figure 6:4

Individual twitches are shown that were recorded at various times throughout a response to  $5.10^{-4}$  M NP during which the oxygen content of the perfusate was varied. The twitches are grouped to show the effects in air, high oxygen, or low oxygen NP solutions with the position of each respective twitch being indicated by the corresponding letter in the complete experimental trace above. As before, the change of perfusate and oxygen tension are indicated by the bars above the trace. The differentiated tension traces are shown below the corresponding twitches and clearly show the changes in twitch shape produced by the change in oxygen levels.

The scale bars for the trace represent 0.05mN (vertical) and 2mins.(horizontal) whereas those below represent 0.05mN (twitches), 0.5mN/sec. (differentiated twitches) and 0.5sec.(common to both).



↑ a    ↑↑↑ d    ↑ e            ↑ f    ↑↑↑ h i j            ↑ k    ↑↑↑ l m n o    ↑ p            ↓ q

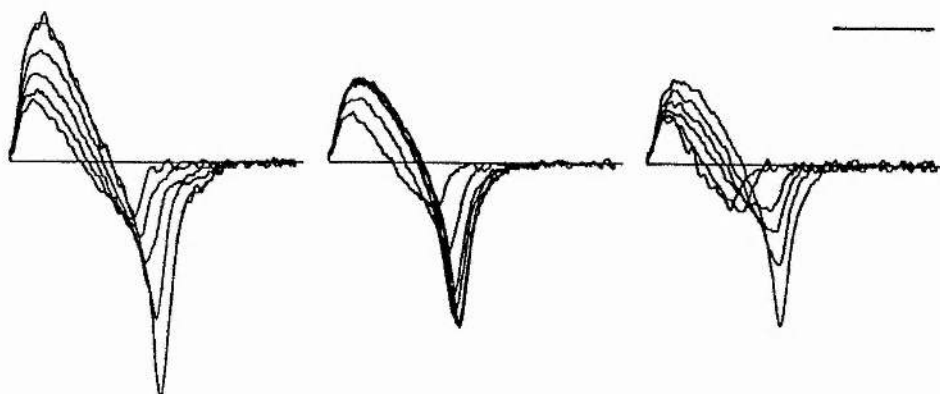
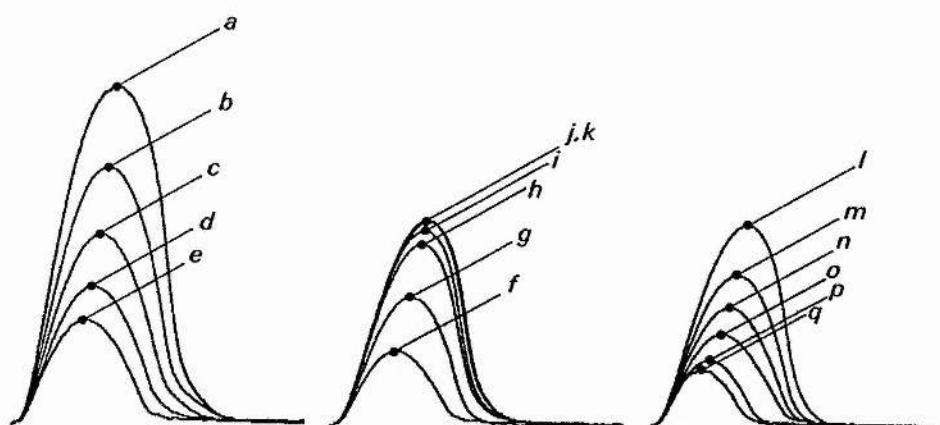
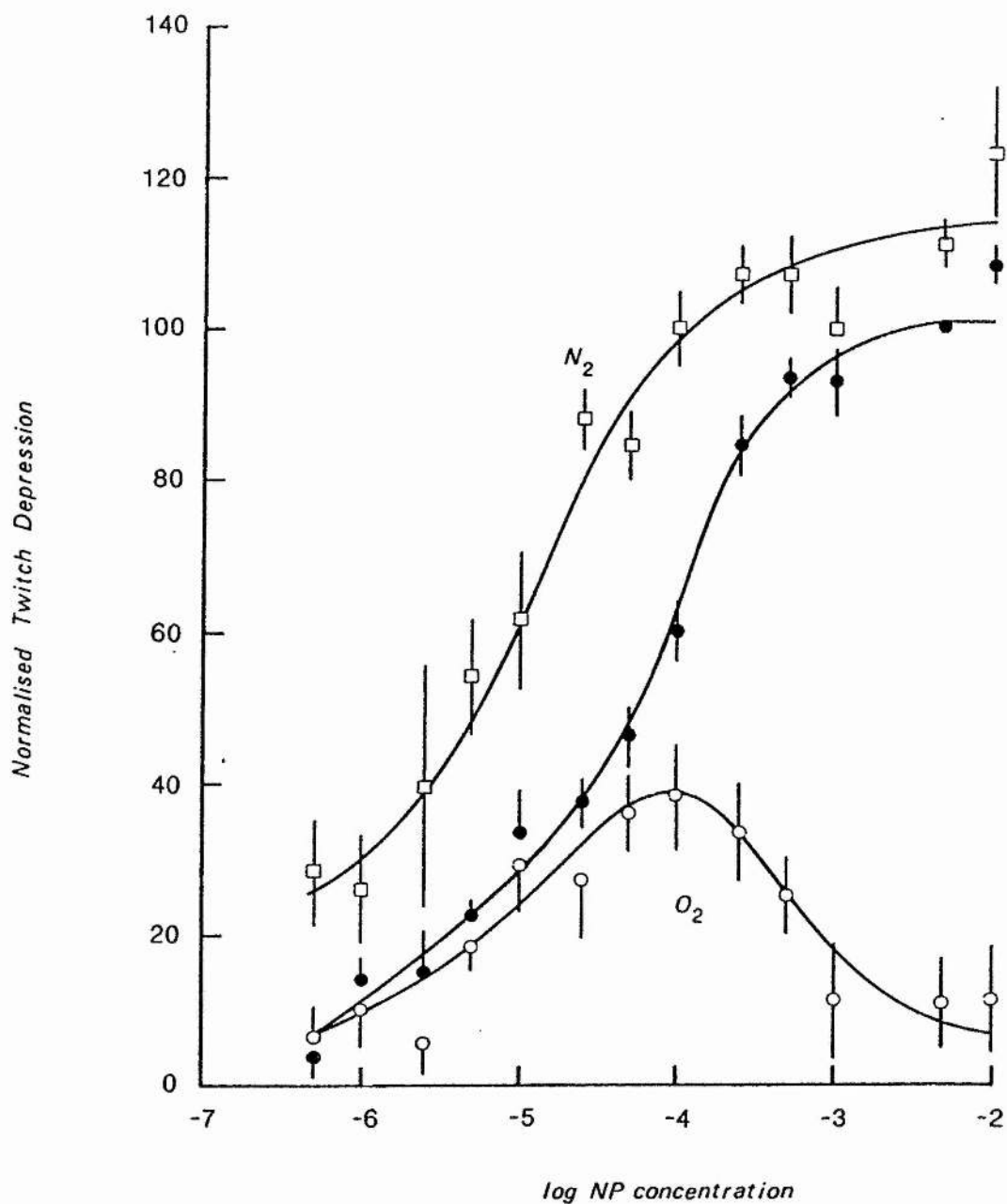


Figure 6:5

The log dose response curves to oxygen ( $O_2$ ), nitrogen ( $N_2$ ) and air saturated NP solutions that were exposed to 20mW 457nm laser light are shown. Each point is the mean value (+ S.E.) of 6-8 experiments. The size of each response is normalised to that produced by  $5 \cdot 10^{-3} M$  air saturated NP in order to compare the relative effects of altering the oxygen level.





to  $10^{-2}M$  at the three oxygen tensions. Each point is the averaged response (+ S.E.) for 6-8 experiments. The curves are normalised to the response recorded using  $5mM$  NP in air saturated solution for ease of comparison. The illumination conditions ( $20mW$ ,  $457,9nm$ ) and flow rate ( $10mls/min$ ) were the same for each oxygen tension. The normal dose response curve (air saturated solutions) is shown by the filled circles and that obtained for  $N_2$ -saturated solutions by the open squares. The effect of reducing the oxygen tension is to produce an upward and leftward shift in the dose response curve, indicating that the efficacy of the perfusate is enhanced. This result is as expected if the physiologically active agent is nitric oxide. However, by the same reasoning, the high oxygen curve should be displaced to the right. Instead, increasing the oxygen tension for  $[NP]'s < 10^{-4}M$  had little effect, as the dose response curve up to this point is indistinguishable from the air saturated (normal) curve. As the  $[NP]$  is increased, the response in high oxygen becomes progressively smaller such that the twitch tension eventually recovers back to its control value at  $10^{-2}M$ . This results in an unusual bell shaped log dose response curve.

#### **Evidence that Aq is not the physiologically active agent**

The above results clearly argue against a physiological role for Aq. This conclusion is based on the following lines of evidence.

First, where it was possible to measure  $[Aq]'s$  directly (ie. at  $[NP]'s > 10^{-4}M$ ), the results for a given  $[NP]$  were independent of the oxygen tension (see table 6:1), even though the inotropic responses obtained were very different.

Thus, for example at  $10^{-3}$  M NP the peak twitch depressions for air,  $N_2$ -, and  $O_2$ - saturated solutions were 63, 72 and 8 % respectively. Although there was no significant difference in the measured [Aq] generated under these three conditions (see table 6:1).

These results also show that the efficiency of the photolytic reaction is not oxygen dependent. In view of this, it is legitimate to estimate [Aq]'s for [NP]'s where it was not possible to measure them directly, as described in the previous chapter. These estimates can then be used to construct log dose response curves for Aq, as shown in figure 6:6(A). Superimposed upon this data are the [Aq] actually measured (filled triangles fig.6:6(A)), which lie at the top end of each curve. Predictably however, the individual log dose response curves do not superimpose but instead retain their separation. The most likely explanation for this is that while [Aq] is a useful indicator of the [NO] generated by the photolytic process, it does not provide a reliable estimate of the amount of free NO remaining in the solution. This instead, will vary directly with [Aq], but will also be inversely related to the oxygen tension ( $[O_2]$ ).

ie.  $[NO]_f = K \cdot [Aq]/[O_2]$

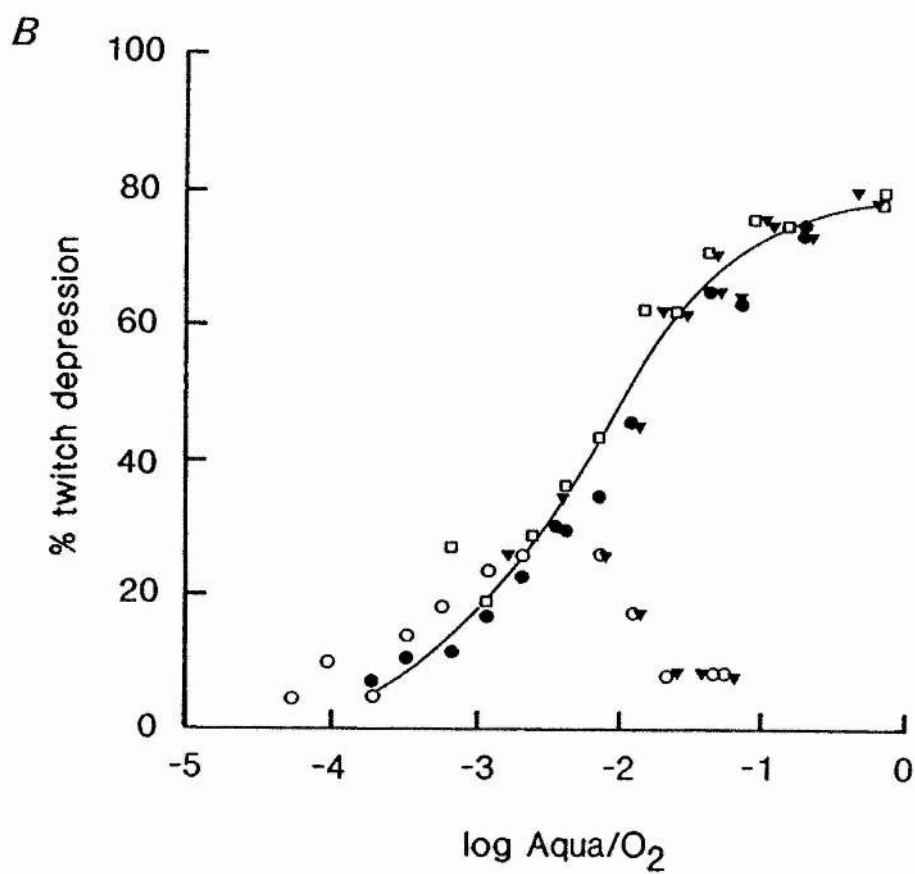
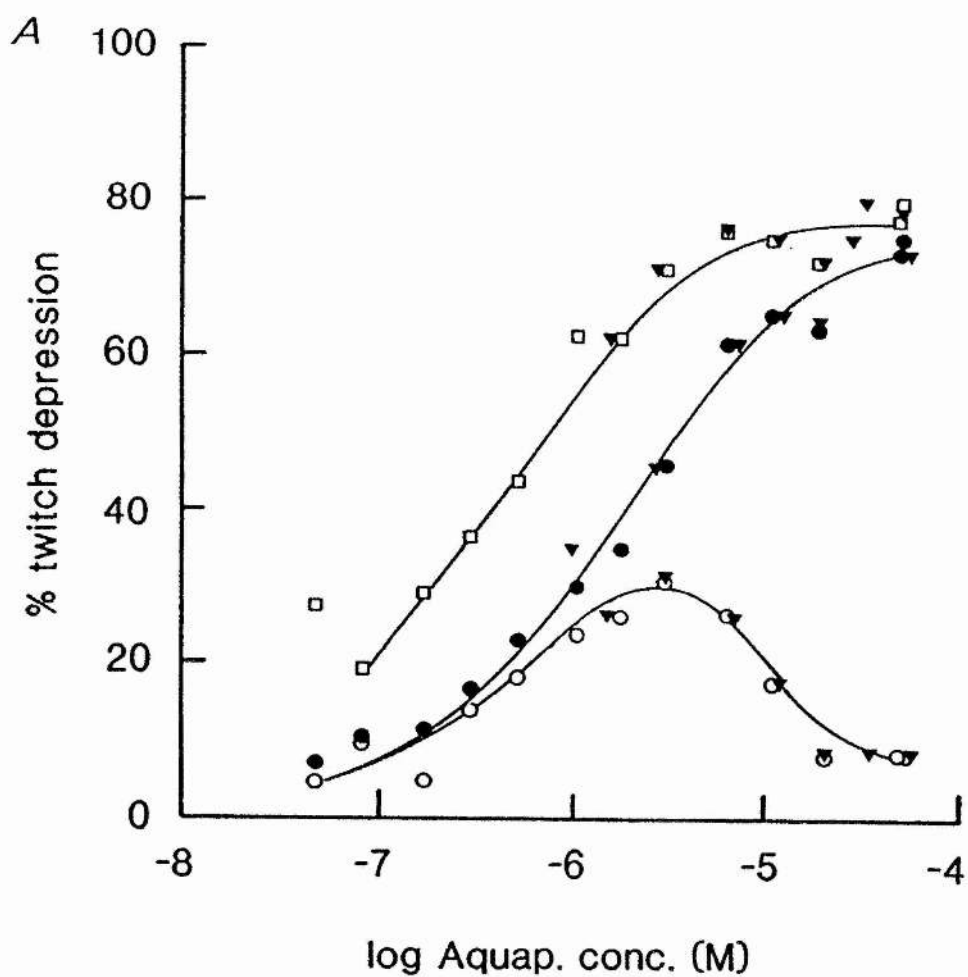
If the physiological response is determined by the [NO] remaining in the solution, then re-plotting the size of the twitch depression as a function of  $[Aq]/[O_2]$  should bring the data together into a single log dose response curve. Figure 6:6(B) shows the results of this analysis. There are two points to notice; First, the  $N_2$ - and air-saturated curves (open squares & closed circles respectively) now exactly

Figure 6:6

The amount of photodegradation of NP was estimated for the data in fig.6:5 using the  $A_q$  estimation procedure previously described. The resulting  $A_q$  log dose response curves are shown in panel A, along with measured  $A_q$  values. Note that the size of the twitch depression is not normalised in this graph and that the error bars are omitted for clarity. The shape and relative position of the curves are very similar to the NP dose response curves which suggest the differences are not due to varying amounts of  $A_q$ .

Panel B shows the same data as above divided by the relative oxygen concentration of each solution. This has the effect of bringing the nitrogen and air saturated curves onto a single curve, but the points for high oxygen at high  $A_q$ , all lie off this curve.

	$[O_2]$
● - air saturated	0.260 mM
○ - oxygen saturated	0.920 mM
□ - nitrogen saturated	0.074 mM
▼ - measured $A_q$ values	



superimpose. Secondly, for  $[NP]'s < 10^{-4}M$ , the data obtained using  $O_2$ -saturated solutions lie along this same curve, whereas for  $[NP]'s > 10^{-4}M$ , the response becomes almost completely suppressed. This last point is especially significant, because it suggests that responses recorded under high  $O_2$  conditions are not solely determined by the  $[NO]$ . Instead, it points to the production of another physiologically active product whose actions oppose those of  $NO$  and effectively protect the twitch. Presumably, this can only happen at relatively high  $[NP]'s$  and high oxygen tensions, but not in lower oxygen tensions (ie air or  $N_2$  saturated solutions)

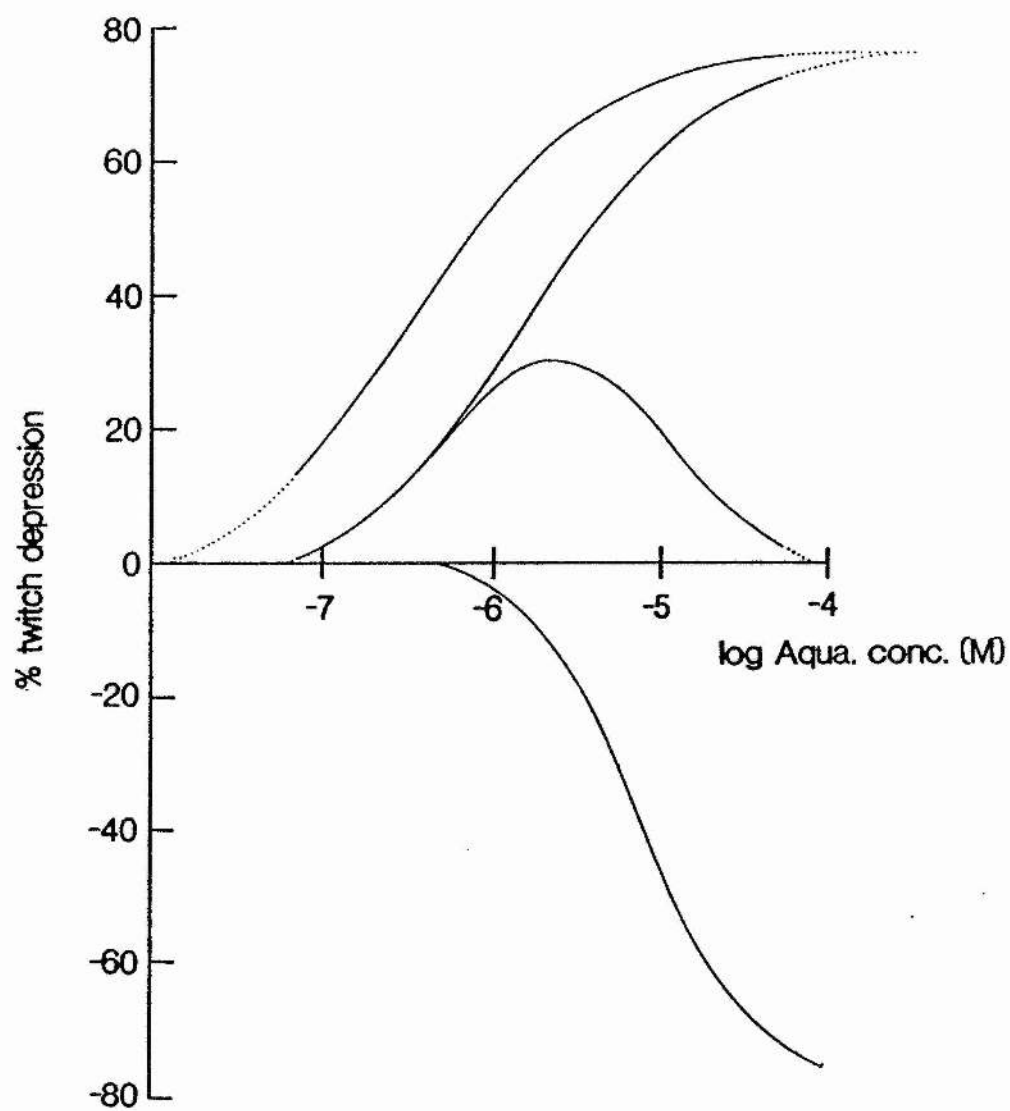
This hypothesis can be understood by reference to figure 6:7. Here, the upper half of the diagram reproduces the dose response curves of fig.6:6(A), the lower half of the diagram shows the difference between the curves obtained using air and  $O_2$  saturated solutions. This can be taken to approximate to the log dose response curve for the hypothetical antagonist, which increases in concentration from a threshold at  $0.5 \mu M [Aq]$  to a maximum effect at around  $100 \mu M [Aq]$ . These concentrations are equivalent to  $[Aq]/[O_2]$  molar ratios of 0.004 and 0.064 respectively.

More direct evidence showing that  $Aq$  is not the physiologically active substance formed during photolysis, comes from experiments in which preparations were perfused with synthetic  $Aq$ , kindly supplied by Dr A.R. Butler of the Chemistry Department. This was found to have no effect on the twitch, even when used at a concentration of  $10^{-3} M$ . It will be recalled (see figs 5:7 & 5:8 ) that this concentration far

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### Figure 6:7

The top half of this diagram is the same as that in figure 6:6 (A) which shows the effects of different oxygen levels on the Aq dose response curve. The bottom half shows the difference curve between the air and oxygen saturated dose response curves. The shape of this curve might suggest a dose response curve for an agent which is produced in high oxygen, is dependent on the Aq concentration and which antagonises the nitric oxide reduction of the twitch. The concentration of this agent is effective above Aq concentrations of  $5 \cdot 10^{-7} \text{ M}$  and can totally antagonise the nitric oxide at concentrations corresponding to  $10^{-4} \text{ M Aq}$ .



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exceeds the  $ED_{50}$  values for the effect on peak tension (1000 x), on  $dP/dt$  (2500 x) and on TTP (240 x), so that we can safely assume that Aq is not involved in the inotropic response.

Evidence that neither nitrite nor nitrate ions are involved in the inotropic response.

Nitrite ( $NO_2^-$ ) and nitrate ( $NO_3^-$ ) are two important products of the secondary reactions which follow the primary photochemical process (see fig.6:1). Some consideration must therefore be given to the possibility that they contribute to the physiological response. The following evidence argues firmly against this;

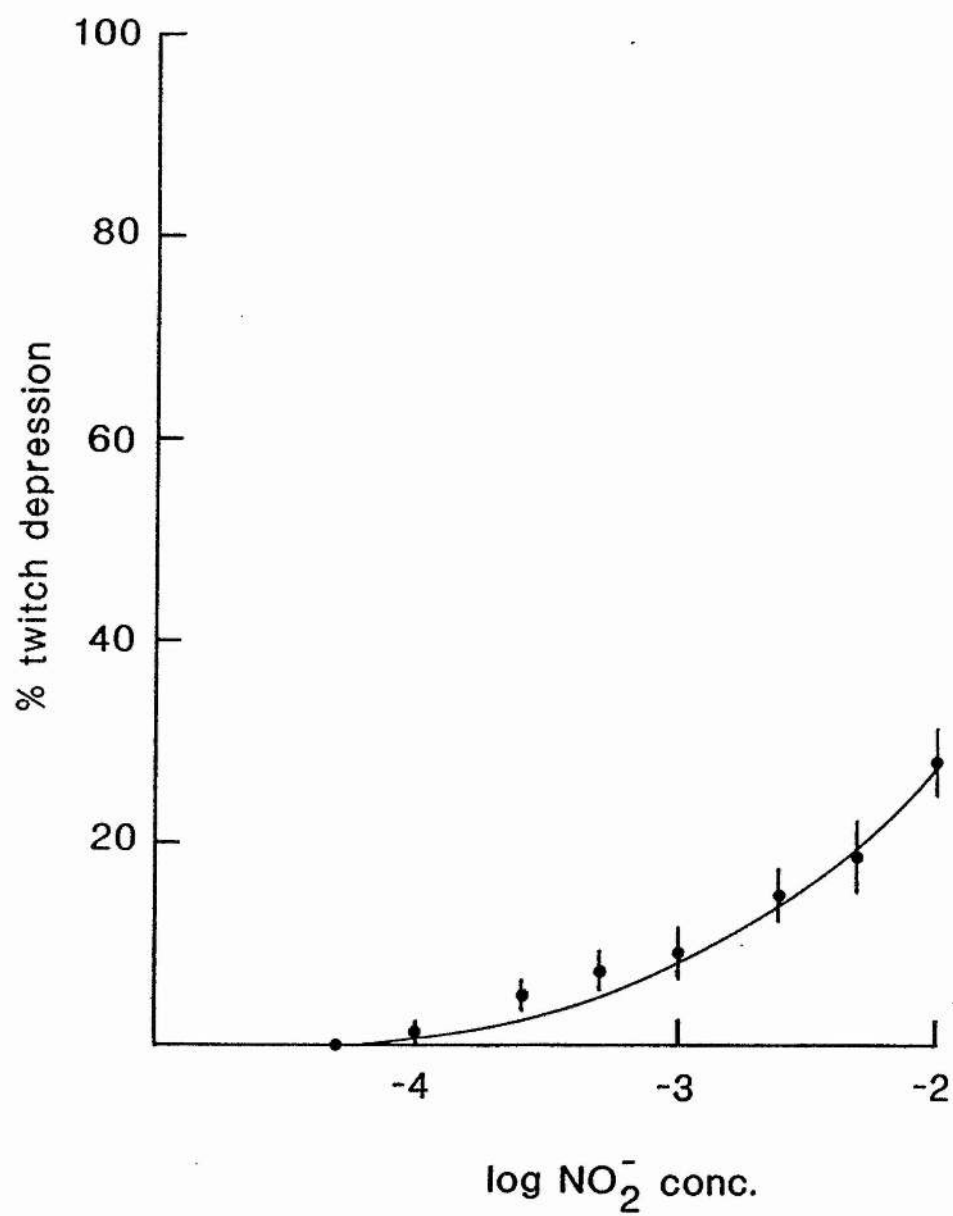
First, the sense in which the responses are affected by changing the oxygen tension is contrary to what one would expect if either  $NO_2^-$  or  $NO_3^-$  were involved. It will be recalled that lowering the oxygen tension inhibits, while raising it favours their formation. Thus if either of these ions were involved, the physiological responses should be enhanced in high oxygen and suppressed in low oxygen conditions, which is the reverse of what is found experimentally. Secondly, experiments were performed in which preparations were directly exposed to different concentrations of  $NO_2^-$  and  $NO_3^-$ . These showed that  $NO_3^-$  is entirely ineffective, although  $NO_2^-$  was found to have a negative inotropic effect at relatively high concentrations as illustrated by the log dose response curve of figure 6:8. The magnitude of the effect is small, producing a 25% depression at 10mM and with a threshold effect at 100uM. It should be emphasised that these concentrations are greatly



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### Figure 6:8

The depression of the twitch caused by various doses of nitrite is shown in the form of a log dose response curve. The concentration required to elicit a response ( $10^{-4}\text{M}$ ) is too large to play a role in the negative inotropic action of photolysed NP.



in excess of those which could be generated photolytically under the conditions of the experiments with NP.

**Effect of agents which influence superoxide availability.**

The results presented so far clearly favour nitric oxide (NO) as being the product responsible for depressing the twitch. This hypothesis was further tested by examining the effect of agents which either stimulate or suppress superoxide ( $O_2^-$ ) formation. Superoxide ions react rapidly with NO to form nitrate. Thus the addition of agents which stimulate superoxide production (eg.  $Fe^{2+}$  ions) should inhibit responses. The outcome is less predictable in the case of substances which suppress superoxide levels (eg. superoxide dismutase or  $Cu^{2+}$  ions). These will only enhance the effect of NP if significant basal superoxide levels are normally present, otherwise they should have no action. The following experiments were made using air saturated solutions of NP illuminated with 20mW light at 488nm.

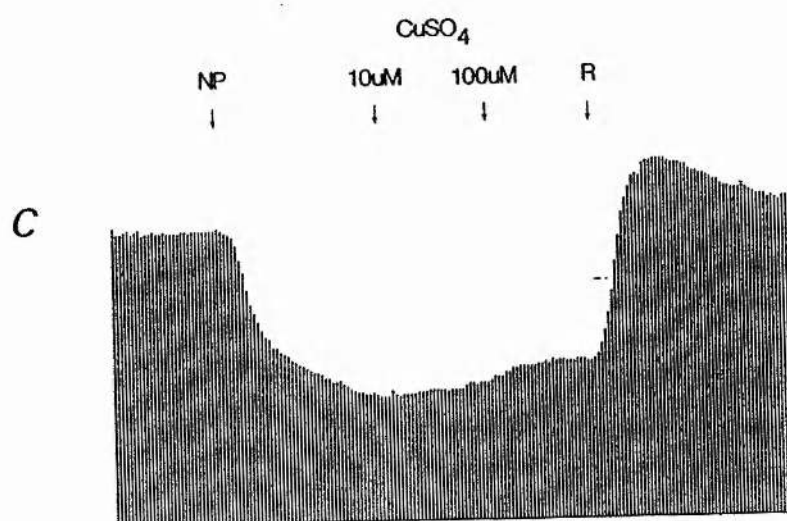
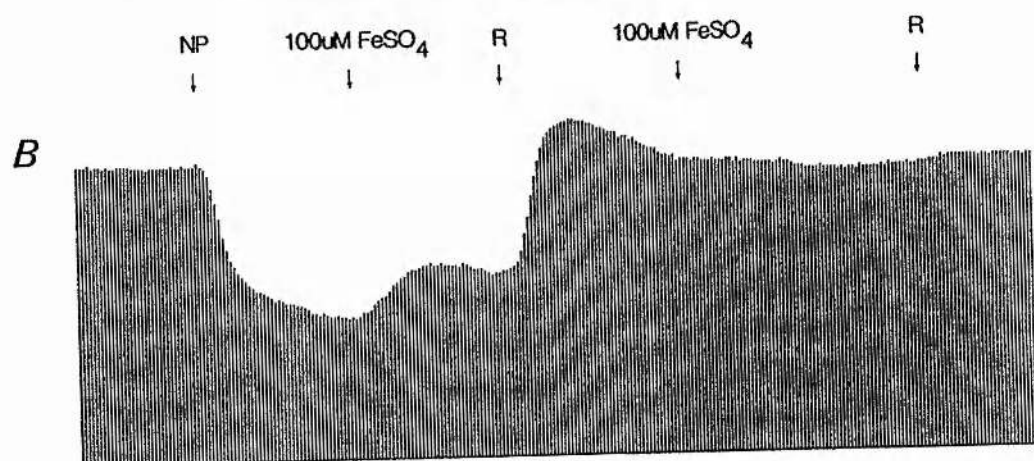
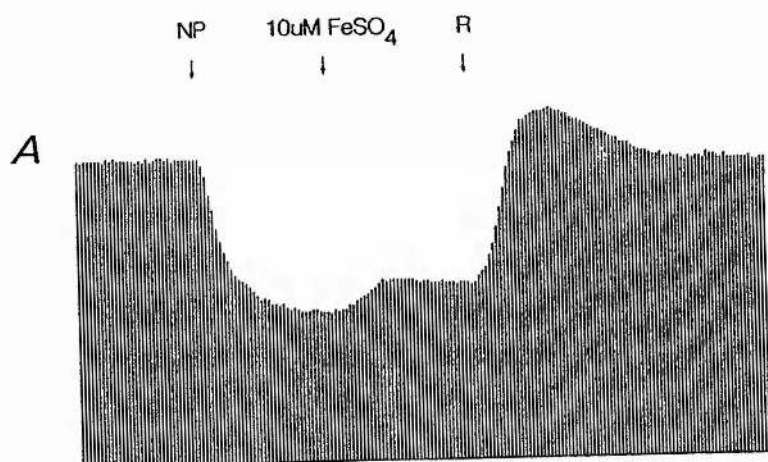
**Effect of  $Fe^{2+}$  ions.** Figure 6:9 (A,B) illustrate the effect of adding 10uM (A) and 100uM (B)  $FeSO_4$  during responses elicited by  $10^{-3}$  M NP. In both cases, the initial depression of the twitch is partially reversed by addition of  $Fe^{2+}$  ions. The 'protective' effect of  $Fe^{2+}$  was dose dependent in that the size of the reversal of the NP response was greater at 100uM than at 10uM. Control experiments showed that  $Fe^{2+}$  added to normal Ringer or unexposed NP had no effect on the twitch (see fig.6:9(B)).

**Effects of  $Cu^{2+}$  ions and SOD.** The effects of adding  $Cu^{2+}$  or SCD to the perfusate is illustrated in figures 6:9(C) and

Figure 6:9

Each panel shows a computer record of the depression in peak twitch tension produced by  $10^{-3}$  M NP perfusate exposed to 20mW 488nm laser light (NP). After the twitch tension had stabilised, the perfusate was changed to NP solution containing either 10uM (panel A) or 100uM (panel B) ferrous sulphate ( $\text{FeSO}_4$ ). The ferrous ions increase the concentration of superoxide ions in solution which causes a partial reversal of the twitch depression. The effect of  $\text{FeSO}_4$  in normal ringer was also tested (panel B) which showed that it had no inherent inotropic action.

A decrease in the superoxide ion concentration was produced by adding copper sulphate ( $\text{CuSO}_4$ ) in a similar manner (panel C). This did not enhance the twitch depression as expected but had no effect or a small antagonistic effect on the twitch depression.



2 min.

.05 mN

6:10(B). These agents would be expected to enhance the action of NP or else not affect it at all (see above). Paradoxically however, the responses were attenuated by both. This is contrary to what was anticipated, although in both cases the effect was small. It may be significant that the protective action of  $\text{Cu}^{2+}$  appears to be dose related as 100uM  $\text{CuSO}_4$  was more effective than 10uM. The reason for this unexpected effect is not known.

In summary, these results preclude a major role for superoxide ions in influencing the physiological responses to photolysed NP, at least when using air saturated solutions. However when the superoxide levels are increased by Ferrous ions, the responses are reduced. This result is consistent with the hypothesis that NO is responsible for depressing contractility. In retrospect, it would have been interesting to have investigated the significance of superoxide at low and high oxygen tensions, but this was not done.

#### Haemoglobin suppresses responses to photolysed NP.

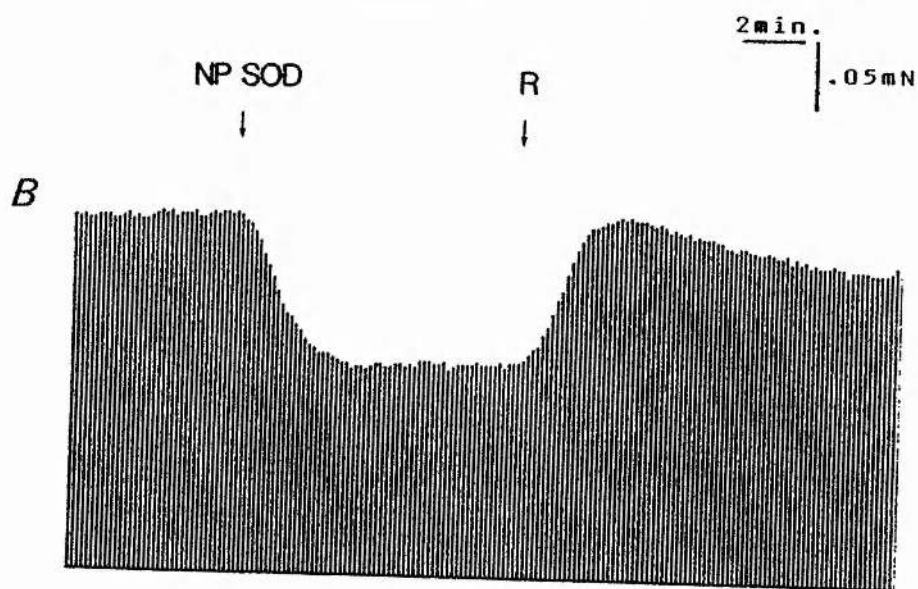
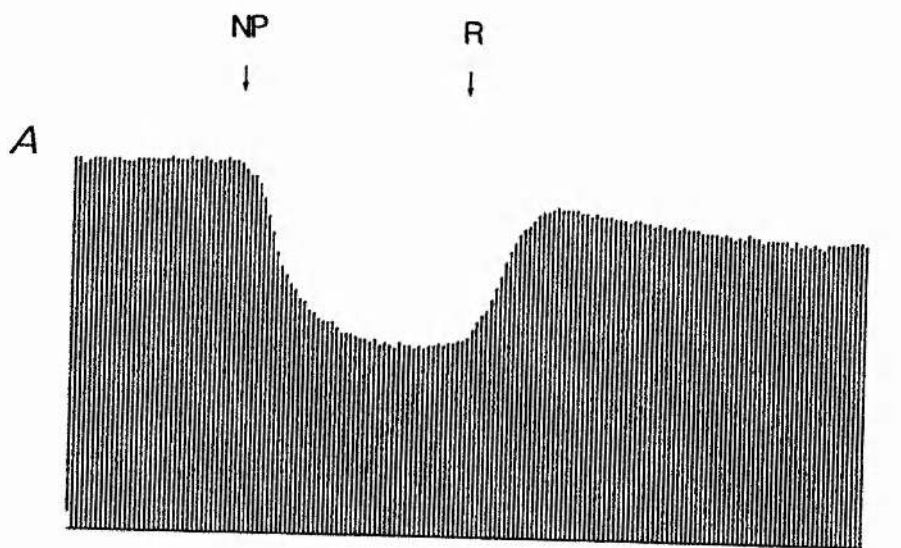
The haem prosthetic group of haemoglobin ( and other haem containing proteins) reacts with NO to form nitrosyl-haemoglobin (Hb-NO). The reaction is extremely rapid and the product is stable under normal conditions (Antonini and Brunori, 1971). The absorption spectrum of haemoglobin is changed on binding NO, and this can be used as a means of identification. Furthermore, the unpaired electron associated with the bound NO renders Hb-NO paramagnetic, so that its formation can be confirmed by means of electron paramagnetic resonance(EPR) spectroscopy.

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### Figure 6:10

Panel A shows the control depression of the twitch (50% reduction in the twitch) caused by  $10^{-3}$ M NP illuminated by 20mW 488nm laser light.

Panel B shows the same response in the presence of 30 units of the enzyme superoxide dismutase (SOD). This enzyme catalyses the conversion of superoxide ions into hydrogen peroxide and so reduces the levels of  $O_2^-$ . SOD caused a slight reduction in the extent of the twitch depression (42% twitch reduction).





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In the experiments described below, Hb is used as a direct means of 'scavenging' free NO generated by the photolytic process. The results will show that the physiological responses are attenuated by Hb in a dose dependent manner.

It was not practicable to identify Hb-NO formation under precisely the same conditions as were used for studying the physiological effects of Hb. EPR spectroscopy is relatively insensitive and greater concentrations of both Hb and NP were required in order to generate adequate signals. However, Hb-NO production was detected in separate experiments, which in all other aspects exactly simulated the normal conditions.

ProtocolThe experiments necessitated some minor changes to the apparatus. There were two problems to overcome. First, Hb strongly absorbs light at wavelengths  $< 600\text{nm}$ ; and secondly, Hb-NO can be made to photodissociate, liberating free NO if exposed to strong light. Both problems were circumvented by keeping the NP and Hb solutions separate during photolysis, and only mixing them immediately prior to entering the muscle chamber. NP solutions flowed through the exposure tube (route A) and were exposed to  $20\text{mW}$  of  $488\text{nm}$  light in the usual way. Hb containing solutions were made to flow through a separate tube (route B) by-passing the exposure tube. The two routes were united through a T-junction, located close to the exit of the exposure tube, which served as a mixing chamber for the two solutions. The flow rates through each route were carefully adjusted so that a 50:50 mixture was obtained. This

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was checked periodically by comparing the absorbances of the chamber effluent and the initial (unmixed) solutions. Solutions of NP and Hb were made up at twice the required concentration to allow for dilution during mixing in the inlet tube to the muscle chamber.

Figure 6:11 (A,B&C) shows three responses to 1mM NP exposed to 20mW of light at 488nm. After allowing the twitch to stabilise, the perfusate in route B was switched from normal Ringer solution to Ringers solution containing Hb. Again the twitch was allowed to reach a new steady state before switching both perfusates to normal Ringers solution to allow the preparation to recover. These recordings show that the addition of Hb serves to diminish the NP response. The effect is clearly dose dependent, increasing with higher [Hb]'s until the effect of NP is completely suppressed in the presence of 2.5uM Hb (see fig.6:11(C)).

The results from a series of experiments similar to those illustrated in figure 6:11 are summarised in figure 6:12. The filled circles show the peak twitch tension as a fraction of the control value in the presence of NP alone. The open circles show the steady state twitch tensions recorded in the presence of Hb and NP. Concentrations of Hb < 0.1uM have no effect on the response, but above this it becomes progressively reduced until complete recovery of the twitch is seen for [Hb]'s > 2.5uM.

### Spectroscopic identification of Hb-NO formation.

Hb avidly binds NO to form nitrosyl haemoglobin which

## Chapter 6

### Figure 6:11

Each panel shows the depression of the peak twitch tension induced by  $10^{-3}$  M NP exposed to 20mW 488nm laser light. After the response had stabilised, 0.1uM (panel A), 1.0uM (panel B) or 2.5uM (panel C) haemoglobin (Hb) was added to the perfusate as described in the methods. Hb antagonises the negative inotropic effects of exposed NP in a dose dependent manner.

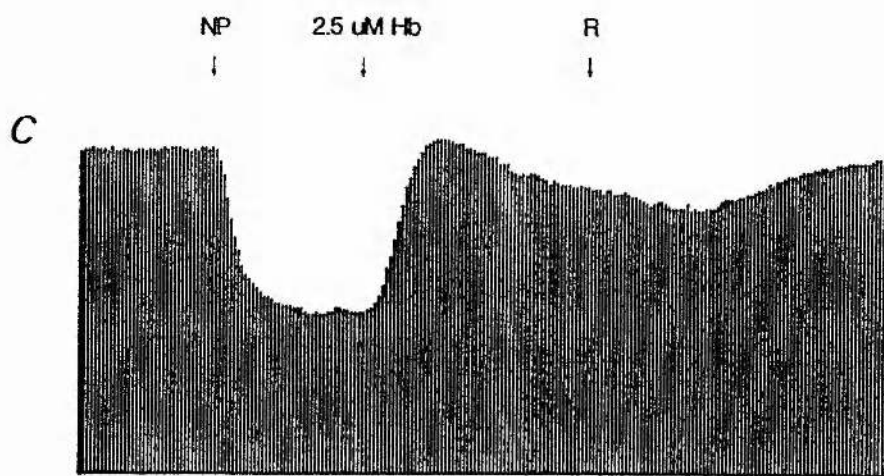
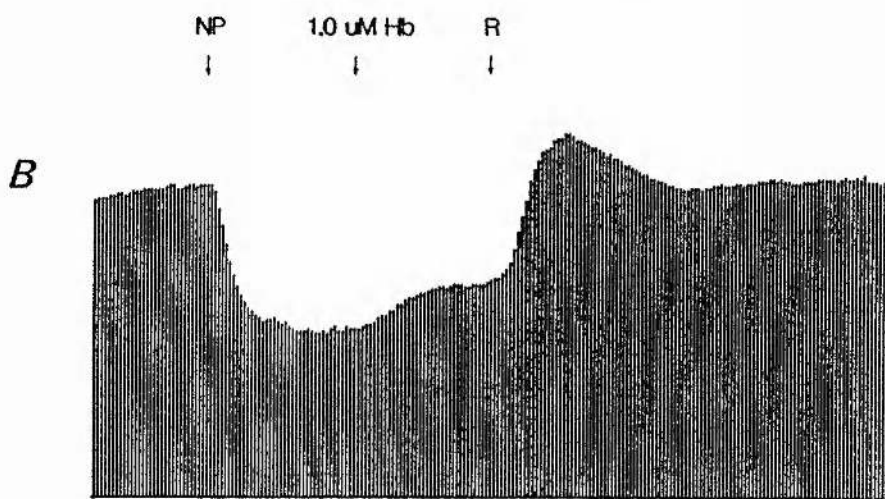
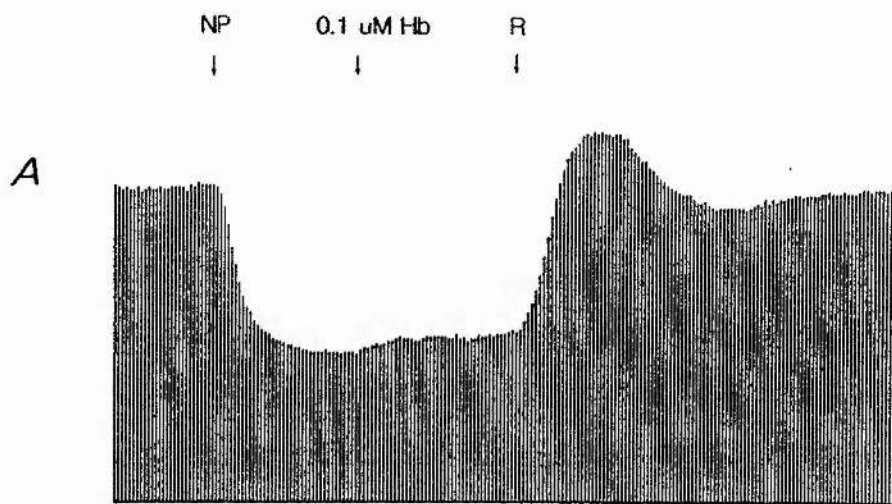
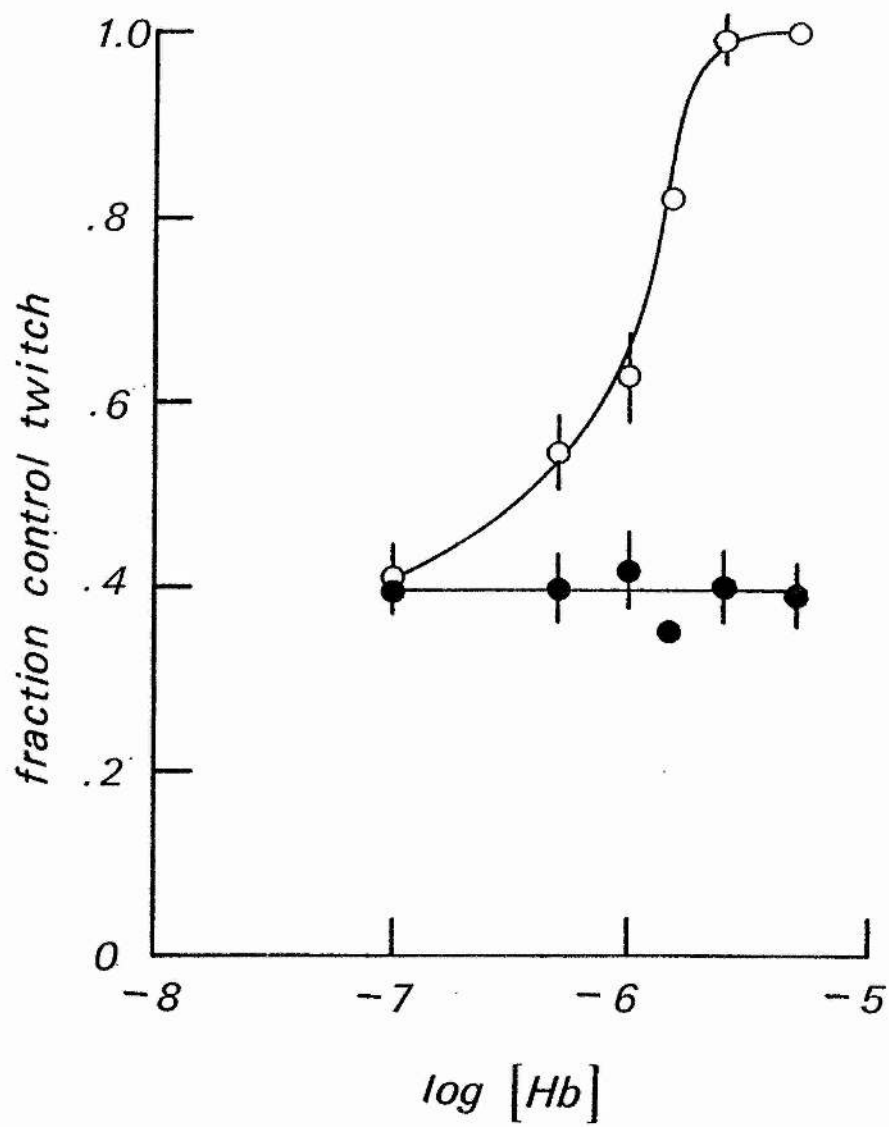


Figure 6:12

The size of the remaining twitch in  $10^{-3}\text{M}$  exposed NP perfusate in the absence (closed dots) and presence (open dots) of varying concentrations of Hb, is expressed as a fraction of the control twitch and plotted against the Hb concentration. Increasing the Hb concentration above  $10^{-7}\text{M}$  causes a dose dependent reversal of the twitch depression, until at concentrations higher than  $2.5\text{ }\mu\text{M}$  it can totally reverse the effects of  $10^{-3}\text{M}$  NP.



can be identified spectroscopically. The quantities of NP and Hb employed in the experiments described above were not sufficient to generate detectable EPR spectra and so the formation of Hb-NO was investigated in a separate series of experiments which were designed to simulate the conditions normally used.

**Protocol** All solutions were made up in  $N_2$ -flushed 100mM phosphate buffer at pH 7.0. A solution of NP (50mM) was made to flow through the exposure tube and illuminated with 2W laser light at 488nm. The high intensity of illumination was used to maximise NP photolysis. The exposed NP solution was then mixed with an equal volume of Hb (1mM) containing dithionite (DTN; 2mM). DTN was included to ensure that Hb was present in its deoxy form (see results). The resulting solution was allowed to stand for 5min in a nitrogen atmosphere, and then diluted 10 fold with deoxygenated phosphate buffer. The final solution therefore contained Hb (50uM), exposed NP (2.5mM) and DTN (0.1mM). The absorption spectrum (350-750nm) was then recorded against 2.5mM NP without Hb as a blank, in a Cecil dual-beam spectrophotometer interfaced to a BBC microcomputer. This procedure was repeated but this time NP was not exposed to light.

Standard samples of oxy- and nitrosyl-haemoglobin were prepared for comparative purposes. Nitrosyl-Hb was synthesised in one of two ways; either by adding excess sodium nitrite to Hb containing DTN, or by bubbling Hb and DTN with NO gas. OxyHb was prepared by dissolving Hb in oxygen saturated phosphate buffer without the addition of DTN.

EPR spectroscopy was performed on a sample of the

exposed NP and Hb, prepared as described above. Unphotolysed NP was first removed by putting the solution through a Sephadex G-25 column under anaerobic conditions. The fraction containing Hb was collected and aliquots were frozen in thin quartz tubes. EPR spectra were then recorded at 17°K.

**Identification of nitrosylhaemoglobin by absorption spectroscopy.** Figure 6:13 (A,C&E) shows the form of the absorption spectrum of deoxyHb. A and C are produced by Hb alone whereas E also contains unexposed NP. The dominant feature is the single, broad maximum centred at 555nm. This region of the spectrum changes in a characteristic manner on ligand binding: the single peak at 555nm splits into two sharper peaks. This is illustrated in B and D, which shows the absorption spectrum of standard samples of oxyHb and Hb-NO respectively. The spectrum for exposed NP and Hb is shown in F. This bears a close resemblance to the standard Hb-NO spectrum (D) with the twin peaks occurring at the same wavelengths (545nm & 570nm). The separation between the peaks is less clear but this may be due to the presence of unbound Hb.

These changes are illustrated more clearly by computing the difference spectra for each of the ligands. These are shown in fig.6:14. The area of interest lies between 500 and 600nm. The top two difference spectra are produced by subtracting the spectrum of Hb alone from oxyHb (B-A; top trace) and from Hb-NO (D-C; middle trace). The lower trace shows the difference between Hb and the product formed by mixing exposed NP and Hb (F-E).

The shapes of these difference spectra are similar, in



## Chapter 6

### Figure 6:13

Shown here are the absorption spectra from 430-750 nm wavelength (space between each dot is 20nm), for the solutions described below. All solutions made up in 100mM phosphate buffer, pH 7.0.

A & C- 50uM deoxygenated Hb in 0.2mM dithionite (DTN) read against buffer.

B - 50uM oxygenated Hb read against buffer.

D - 50uM deoxygenated Hb, 0.1mM DTN and 1mM sodium nitrite read against buffer.

E - 50uM deoxygenated Hb, 0.1mM DTN and 2.5mM unexposed NP read against 2.5mM unexposed NP.

F - 50uM deoxygenated Hb, 0.1mM DTN and 2.5mM NP that was exposed to 200mW 488nm laser light in the exposure tube (flow rate; 7mls/min) read against 2.5mM photoexposed NP.

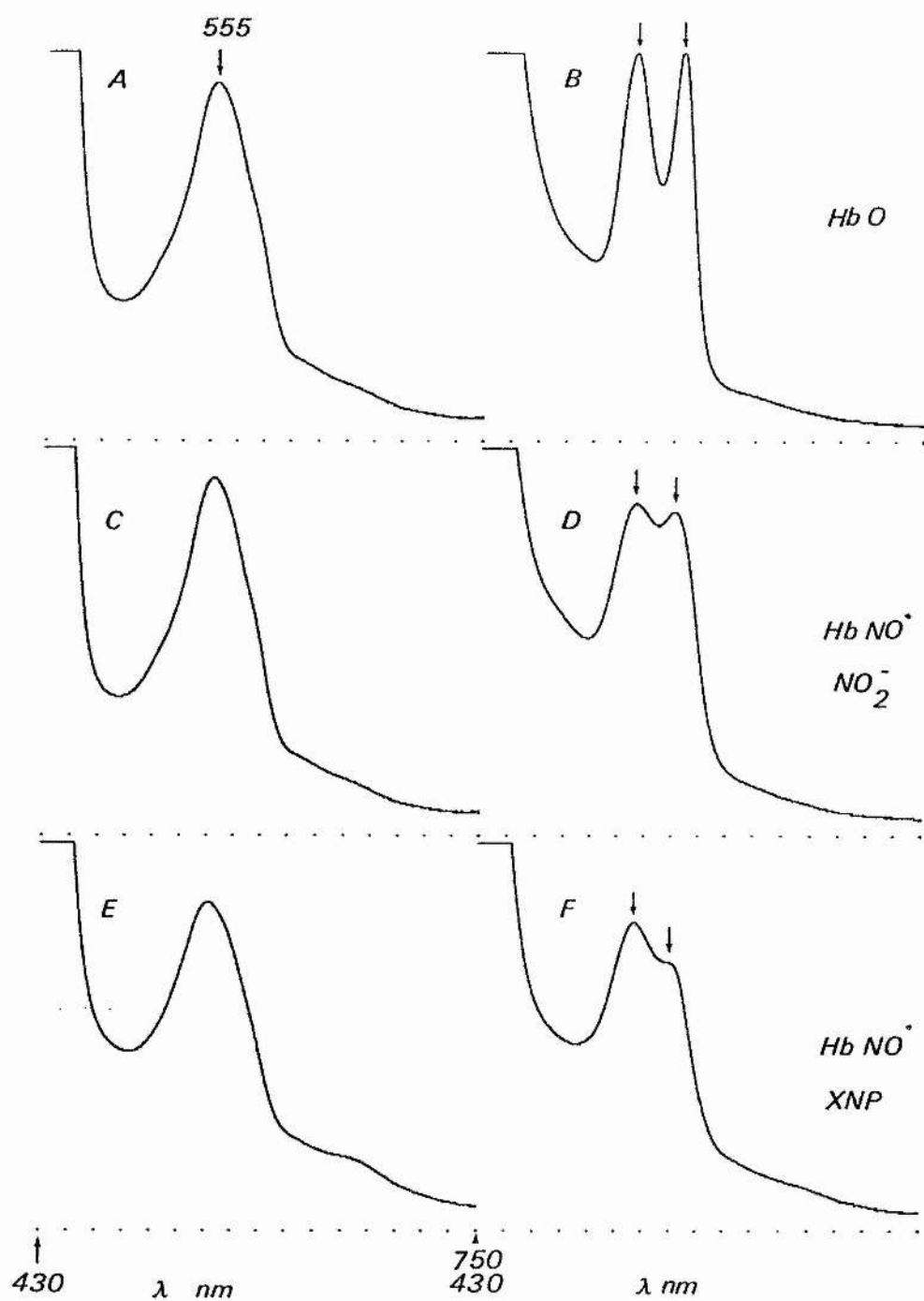


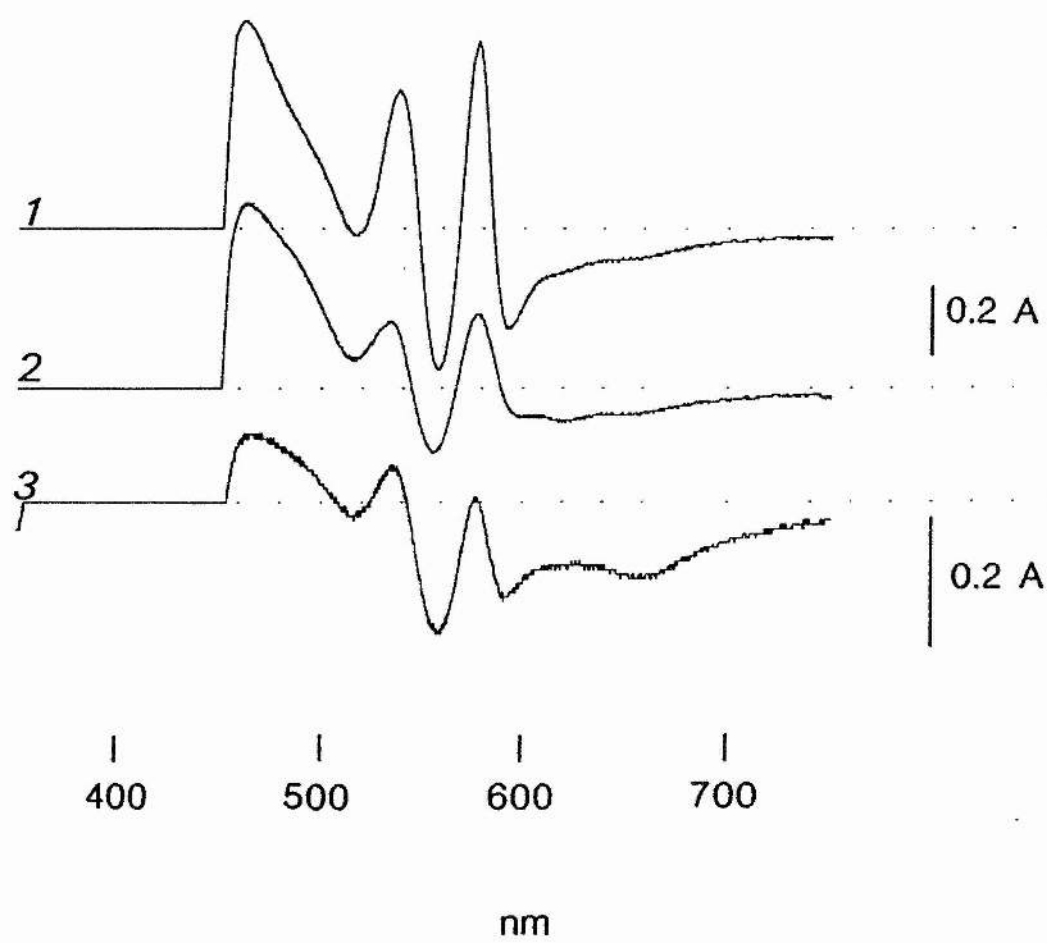
Figure 6:14

The difference in the preceding absorption spectra were calculated to show the change in the absorption spectra of Hb upon combining with oxygen (1), or nitric oxide (2 & 3).

Trace 1 - difference between oxygenated (HbO) and deoxygenated Hb ( B-A ; fig.6:13 )

Trace 2 - difference between standard NO-Hb and deoxygenated Hb ( D-C ; fig. 6:13)

Trace 3 - difference between NO-Hb produced by NP photolysis and Hb ( F-E ; fig. 6:13). (N.b. this trace is at twice the gain of the other two)



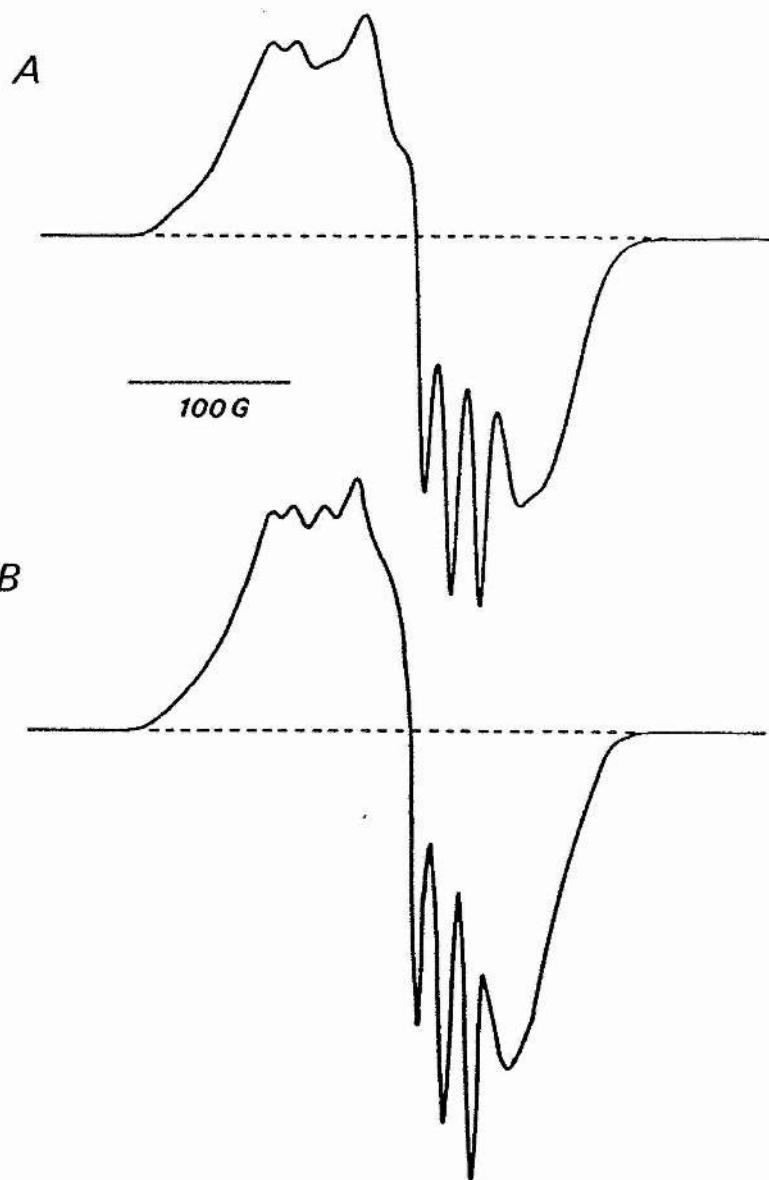
that each comprises two peaks on either side of a trough. However, there are subtle differences between them which are of significance. The position of the first (left) maxima is located at 537nm for oxyHb, but at 533nm for Hb-NO and also for the mixture produced from exposed NP and Hb. The position of the minimum also differs, as it occurs at 557nm for oxyHb but at 555nm for the other two spectra. These minor differences between the maxima and minima for the two ligands agree with spectra published in the literature (Antonini and Brunori, 1971).

Further confirmation that Hb-NO is formed under the experimental conditions used in this study comes from EPR spectroscopy. This method relies upon the single unpaired electron associated with NO which becomes bound to the haem group. Thus, both Hb and oxyHb which lack an unpaired electron are EPR 'silent'. Two EPR spectra are shown in figure 6:15; the lower one is taken from a paper by Trittelvitz, Sick and Gersonde (1972), which shows the EPR signal generated by Hb-NO at pH 5.1 at a temperature of 77 K. The upper spectrum is from the same sample of exposed NP and Hb that gave the absorption spectrum F in fig.6:13. It closely resembles the lower spectrum, showing very sharp 'hyperfine splitting', characteristic of the highly protonated (low pH) form of Hb-NO.

Figure 6:15

Spectra A is the EPR spectra for the same solution that produced spectra F in figure 6:13. A sample of this solution was prepared for EPR spectroscopy as described in the methods and read at 17 K, scan 1000G, midscan 3500G.

Spectra B shows the EPR spectra of NO-Hb as recorded by Trittelvitz, Sick & Gersonde (1972) at pH 5.1, 77 K, drawn on the same scale.



Discussion

Photolysis of NP results initially in the expulsion of NO and the formation of pentacyanoferrate. This event initiates a complex reaction sequence that leads to the formation of several ionic species, some of which have been investigated as potential causative agents for the inotropic effects of 'NP' on frog trabeculae. The results of these experiments preclude a major role for  $\text{NO}_2^-$ ,  $\text{NO}_3^-$  and Aq, and instead strongly implicate NO as being the physiologically active agent formed during photolysis. The main evidence in support of this claim is based on the observation that substances which are known to react with NO, and therefore act as NO scavengers, consistently depress responses to photolysed NP. These include dissolved (molecular) oxygen ( $\text{O}_2$ ), superoxide anions ( $\text{O}_2^-$ ) and haemoglobin. Under 'normal' (air saturated solutions) conditions, the most important endogenous scavenger is  $\text{O}_2$ . Thus, the use of nitrogen flushed solutions to decrease the oxygen tension, greatly potentiates the response. The  $\text{ED}_{50}$  value obtained using  $\text{N}_2$ -saturated solutions is approx. 10 fold smaller than that for air saturated solutions (fig.6:5). In contrast, there is little evidence for the involvement of superoxide in modulating the effects of 'NP' at normal oxygen tensions, because neither  $\text{Cu}^{2+}$  ions nor SOD are able to potentiate responses. Indeed, both were found to antagonise the effects of NP, if only to a small degree. On the other hand,  $\text{Fe}^{2+}$  ions which stimulate superoxide formation at normal oxygen tensions, significantly attenuate responses. This implies that superoxide could play a more significant



role at very high oxygen tensions. This point is referred to again later.

The most important evidence in support of an essential role for NO comes from experiments in which Hb was used as an exogenous scavenger for NO. The results from these are entirely consistent with the hypothesis that NO is directly responsible for the effects on the twitch. It will be recalled that different concentrations of Hb were added during the responses evoked by 1mM NP exposed to 20mW laser light at 488nm. This produced a dose related attenuation of the response, with a threshold effect at 0.1uM, which increased sigmoidally until it entirely reversed the effects of NP at concentrations greater than 2.5uM (fig.6:12). Since there are four haem groups per molecule of Hb (tetramer), and each haem group can react with a single molecule of NO, then these concentrations have the capacity to bind 0.4uM and 10uM NO respectively (Antonini and Brunori, 1971). Spectrophotometric measurements of Aq production under the conditions of these experiments (as an indication of NO production) returned a mean value of 12uM (n=5) which is in good agreement with the [Hb] required to completely suppress the response.

Spectroscopic measurements have shown that NO is liberated during photolysis and that it can bind to Hb under conditions which simulate as closely as possible the experiments referred to above. The product nitrosyl haemoglobin (Hb-NO) has a characteristic absorption spectrum which differs significantly from that of Hb. Deoxy-Hb has a single, broad absorbance peak with a maximum at 555nm, but on binding NO this splits into two, centred at 545nm and 575nm

(Antonini and Brunori, 1971). The substance generated by mixing exposed NP with Hb has a spectrum which bears a close resemblance to this (cf. fig.6:13 (D & F)), although its precise shape is not identical to standard Hb-NO. However, EPR spectroscopy of this same sample provided incontrovertible evidence for the presence of Hb-NO. Neither Hb nor HbO contain an unpaired electron and are therefore EPR silent such that there can be no ambiguity resulting from contamination of the sample with either of these proteins. The EPR spectrum actually obtained (fig.6:15, upper trace) is virtually identical to that obtained by Trittelviche, Sick and Gersonde (1972) (fig.6:15, lower trace). These authors studied the effect of pH on the EPR spectrum of HbNO at low temperatures. They found that lowering the pH exaggerates the hyperfine lines characteristic of HbNO. The spectrum obtained here was actually generated by a sample dissolved in 100mM phosphate buffer at a nominal (room temperature) pH of 7.0. However, Williams-Smith, Bray, Barber, Tsopanakis and Vincent (1977) have reported that rapid freezing of phosphate buffered solutions for EPR spectroscopy causes a large decrease (2-3 pH units) of the apparent pH. There is little doubt that this situation is inadvertantly simulated here and that the EPR spectrum obtained is the highly protonated form of Hb-NO.

There is other less direct evidence that lends support to the hypothesis that NO is responsible for the 'NP' response. Under certain conditions, NO is a potent stimulator of guanylate cyclase (GC) the enzyme which catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) (Waldman and Murad, 1987). Flitney,

## Chapter 6

Moshiri and Singh (1980a,b) have demonstrated that the negative inotropic effect of NP on frog ventricular muscle is accompanied by substantial increases in intracellular cGMP levels. Until now, this was attributed to a direct effect of the NP anion, but the experiments in question were not conducted in complete darkness and the results of the present study make it virtually certain that some photolysis must have occurred. Indeed, as has been emphasised throughout this study, when adequate care is taken to exclude light, NP has no physiological effect on frog ventricular muscle.

The situation is clearly very different for vascular smooth muscle cells. The smooth muscle relaxant action of NP and the other so-called 'nitrovasodilators', such as glyceryl trinitrate, amyl nitrate, hydroxylamine and azide, are not light dependent. Indeed with the exception of NP, all are photostable and it is common practice when using NP clinically to take care to ensure that it is not exposed to light, either before or during infusion. Nevertheless, all of those agents can cause smooth muscle relaxation; all can stimulate GC; and they all produce a rise in intracellular cGMP levels. There is clear evidence too that the production of free NO is a crucial step in their mechanism of action (Waldman and Murad, 1987). These considerations lead one to conclude that frog ventricular cells must lack the capacity to extract NO from NP, since they can only respond to NP if NO is first liberated photolytically. It is possible to be more specific about the difference between these two cell types, because the ability of smooth muscle to release NO appears to depend upon tissue thiol (SH) groups (Needleman and Johnson, 1973) or

direct interaction with the prosthetic haem group of GC (Craven & DeRubertis, 1978). Part of the evidence for the latter agent is the observed reaction of Hb with NP to form NO-Hb, but this was not seen in these experiments, as intact NP did not react with Hb (Craven & DeRubertis, 1978). Artefactual photolysis of NP may explain this observation, as then NO-Hb would be formed as shown here. These and some related questions, are considered in the following chapters.

Finally, some mention must be made of the paradoxical effects of high oxygen tensions on responses to [NP]'s  $> 10^{-4}$  M, referred to earlier. Most of the experimental data obtained by varying the oxygen tension could be made to fit on a single dose response curve by plotting the twitch depression as a function of the ratio  $[Aq]/[O_2]$ , with the notable exception of that obtained at high  $[O_2]$  (960uM) using [NP]'s  $> 10^{-4}$  M (fig.6:6(B)). These concentrations led to a progressive diminution and eventually a complete suppression of the response to NP giving rise to an unusual bell-shaped dose response curve. Notice here that substantial twitch depressions were seen at corresponding  $[Aq]/[O_2]$  ratios at oxygen tensions of 74 or 240uM. These observations suggest that the combination of high NO and  $O_2$  levels creates a fundamentally different environment which has a 'protective' effect on the twitch. One possibility to be considered is that high oxygen tensions generate greater superoxide levels. Qualitatively, the effect of this would be as seen, to suppress the 'NP' response. However, responses generated by  $[NP] < 10^{-4}$  M should also be suppressed, but this was not so: these data lie on the same dose response curve as that

obtained using air saturated solutions.

Another possibility is that one or more of the products generated by the reaction of NO with  $O_2$  (fig.6:1) acts via an independent cellular mechanism to produce a positive inotropic response. This would then serve to oppose the response normally evoked by NO. There is no direct evidence to support this view. No positive inotropic effect was recorded in any experiments with  $NO_2$  or  $NO_3$  nor incidently with synthetic Aq.

Experiments by Murad, Lewicki, Brandwein, Mittal & Waldman (1981), concerned with the kinetics of GC activation by NO, provide a clue as to what might be happening. These authors showed that treatment of the isolated enzyme with NO leads to activation at low concentrations but inhibition at high concentrations. Their dose-response is therefore bell-shaped. The activation of GC is known to depend critically on its redox state and in particular, on conditions which favour the retention of functionally important SH groups and which also keep the haem iron in its ferrous state. A highly oxidising environment will compromise these requirements, by allowing the formation of disulphide bonds between neighboring SH groups and the oxidation of  $Fe^{2+}$  to  $Fe^{3+}$ , either or both of which would tend to suppress the enzyme. If the action of NO on frog trabeculae is mediated through GC, then such an environment could de-activate the enzyme and lead to a restoration of normal contractility. This is similar to the de-activation seen with isolated GC due to 'overoxidation' (see Gen. Intro.).

## CHAPTER 7

Preliminary investigations into the  
influence of thiols on the nitroprusside response,  
and on the inotropic ability of synthetic  
nitrosothiols on frog ventricle.



Introduction

The previous chapters have shown that nitric oxide generated by photolysis of NP is responsible for its negative inotropic action on frog ventricle. The complete ineffectiveness of intact NP is surprising in view of its action on vascular smooth muscle. The liberation of NO is the necessary intermediate step in the mechanism of action of NP. In smooth muscle this is thought to be mediated through intracellular thiols (primarily cysteine) which cleave the NO group off NP to form short-lived S-nitroso-thiol intermediates. The nitrosothiol intermediates then yield the NO to the prosthetic haem group of guanylate cyclase, which stimulates its activity (Ignarro et al, 1981; see gen. intro.). The inability of frog ventricle to 'denitrate' intact NP could be due to a lack of available thiol groups. The addition of cysteine to intact NP may therefore enable it to induce a negative inotropic response. It has been previously reported that NP + cysteine spontaneously form nitroso-cysteine which is known to be a potent activator of guanylate cyclase (Ignarro et al, 1981). Thiols also have a potentiating effect on NO activation of GC, so that addition of cysteine to photolysed NP might also potentiate the response. Preliminary experiments were conducted to investigate the role of cysteine in the inotropic efficacy of both intact and photolysed NP. The direct action of two synthesised nitrosothiols was also investigated: s-nitrosocysteine and s-nitroso-N-acetylpenicillamine.

Methods

The procedures for recording tension from isolated frog ventricular trabeculae were as described previously. The perfusates were prepared as before, in complete darkness and illuminated by 20mW 488nm laser light during its passage through the exposure tube. The responses to three different solutions was tested; (i) cysteine alone, (ii) cysteine plus intact NP (unexposed) and (iii) cysteine plus photolysed NP. The responses to two nitrosothiols were also tested; nitrosocysteine or S-nitroso-N-acetyl-penicillamine. Nitroso-cysteine was prepared according to Pryor, Church, Govindan & Crank (1982). Nitrogen dioxide ( $\text{NO}_2$ ) gas was added slowly to a solution of cysteine in ethanol or water, and the resulting green solution is separated off and air dried to give deep green crystals. Nitroso-N-acetylpenicillamine was similarly prepared by adding  $\text{NaNO}_2$ , dissolved in water (1M), to N-acetyl-D-L-penicillamine (0.5M) dissolved in acidified methanol, the product being separated and crystallised as before (Field, Dilts, Ravichandran, Lenhert & Carnahan, 1978). The Nitroso-N-acetyl-penicillamine was used as it was more stable than nitrosocysteine. Each nitrosothiol was dissolved in ethanol, and diluted in Ringer just before application to the muscle (unless otherwise stated). In all cases an appropriate ethanol 'blank' was tested to determine the effects of the vehicle.



### Results

The results will show that cysteine, NP + cysteine and the synthetic nitrosothiols (nitrosocysteine and s-nitroso-acetylpenicillamine) all affect twitch contractions. Nitrosocysteine and nitroso-acetylpenicillamine produced the most consistent effects: both were found to depress the twitch. The effects of cysteine whether tested alone or in combination with NP (intact or photolysed) were more variable and appeared to depend upon the previous history of the preparation.

#### Effects of cysteine alone

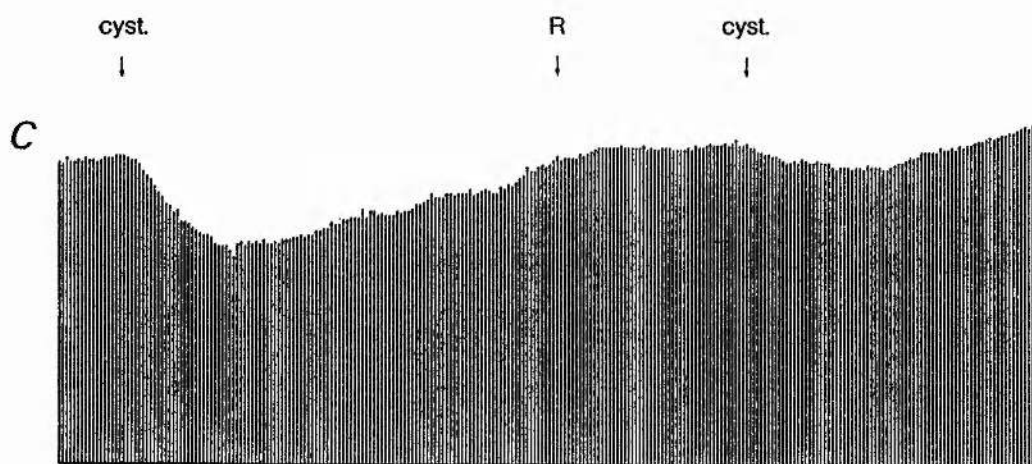
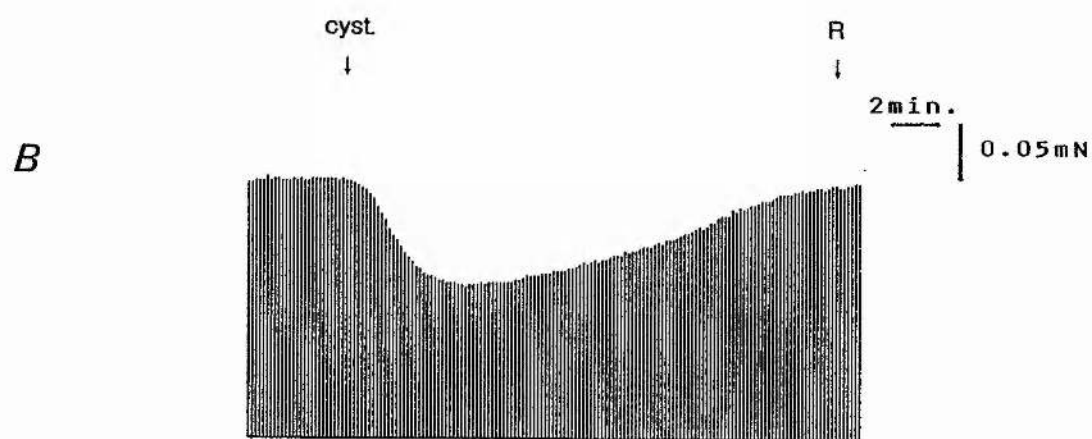
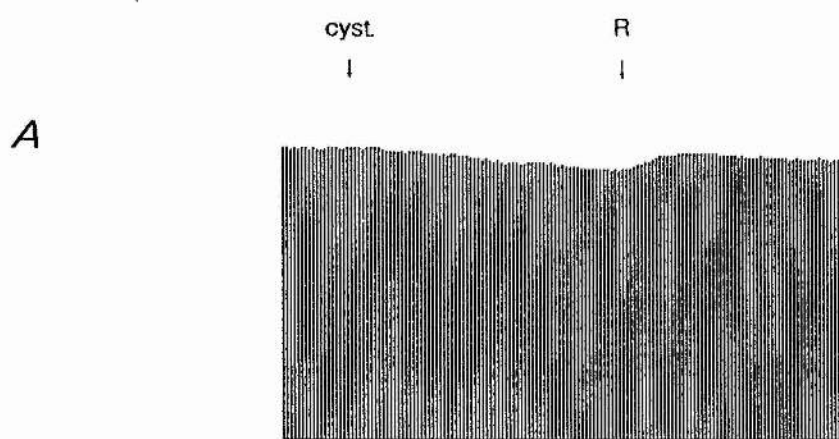
Cysteine (5mM) alone had a variable effect on the twitch, depending upon the time it was tested during the experiment. Some responses are shown in figure 7:1. Cysteine alone did not appreciably affect the twitch when it was applied at the start of the experiment (panel A). This was its most consistent action. However, later during the experiment, after the muscle had been allowed to recover from responses to either photolysed NP, or photolysed NP + cysteine or synthetic nitrosothiols, cysteine induced a large depression of the twitch (panel B). The effect was transient, showing spontaneous recovery, and its magnitude was found to depend upon the severity of the twitch depression elicited previously. This effect is illustrated by reference to panels B & C in figure 7:1. The response shown in panel B was larger (42% depression) than that in panel C (28% depression). The response in B was recorded after the preparation had recovered from a 70% depression induced by nitrosocysteine (see later)

Figure 7:1

Panel A shows the effect of perfusing 5mM cysteine in normal Ringer at the start of an experiment. Cysteine has little effect on the size of the peak twitch tension (7% twitch reduction)

Panel B shows the same solution of cysteine (5mM) applied to the muscle at the end of the same experiment as in A. Here, cysteine causes a marked transient depression of the twitch (45% twitch reduction) that spontaneously recovers back to control levels. In between these applications, the muscle had been subjected to various negative inotropic agents (ie. photolysed NP, NO-thiols) but had fully recovered in normal Ringer before the second application of cysteine.

Panel C shows the effect of applying 5mM cysteine to the muscle at the end of an experiment, which again caused a transient reduction in twitch tension (28%). After the twitch had spontaneously recovered, a second application of the same cysteine solution failed to elicit the same effect (only 7% twitch reduction).



whereas C was after a 40% depression produced by nitroso-penicillamine. Note that both cysteine responses show spontaneous recovery. The recovery rates are similar, with half times of 8mins (B) and 7mins (C). After recovery in normal Ringer solution, a subsequent application of cysteine fails to produce the same effect: in C, only a 7% reduction in peak tension, and again showing spontaneous recovery.

#### **Responses to cysteine in the presence of intact NP**

Intact (unexposed) NP does not affect the responses on fresh preparations. However, it was able to prevent or reverse the depression caused by cysteine applied at a later time in the experiment, after preparations had recovered from prior responses. Figure 7:2 shows the effect of applying NP (5mM) during a cysteine response. This was recorded after recovery in normal Ringers solution from prior exposure to photolysed NP. Cysteine produces a small reduction in twitch tension, as before. The addition of NP (prior to spontaneous recovery) accelerates the recovery process.

These observations would suggest that cysteine is not able to react with intact NP to release NO or to form nitrosocysteine, since either (or both together) would have produced a greater twitch depression (see later)

#### **Effects of cysteine on responses to photolysed NP**

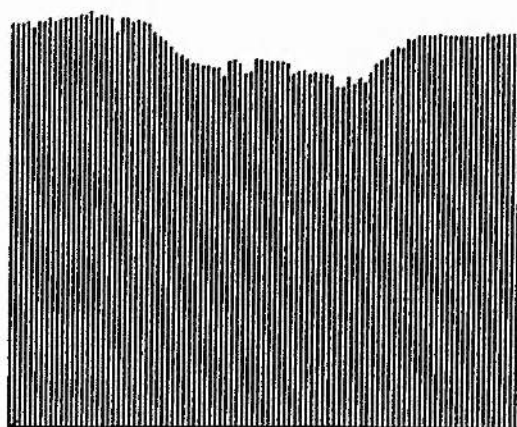
Two kinds of experiment were performed. In the first, a response to photolysed NP was produced and sufficient time was allowed for the twitch tension to reach its new (depressed) steady state. The perfusate was then switched over to NP + 5mM cysteine. In the second, a control response to photolysed NP was recorded and the preparation allowed to

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### Figure 7:2

The effect of intact nitroprusside in the presence of cysteine was tested on frog ventricular twitch tension. Cysteine (5mM) was added alone to the perfusate which caused a slight decrease in twitch tension (12%). The perfusate was then changed to a cysteine solution containing  $5 \cdot 10^{-6} \text{M}$  intact NP. The twitch tension quickly recovered back to control levels in the presence of intact NP.

cyst.      5uM NP+ cyst.



0.05mN

2min.

## Chapter 7

recover in normal Ringers solution before taking the effects of cysteine + exposed NP into consideration. The results obtained were essentially similar to the other protocol.

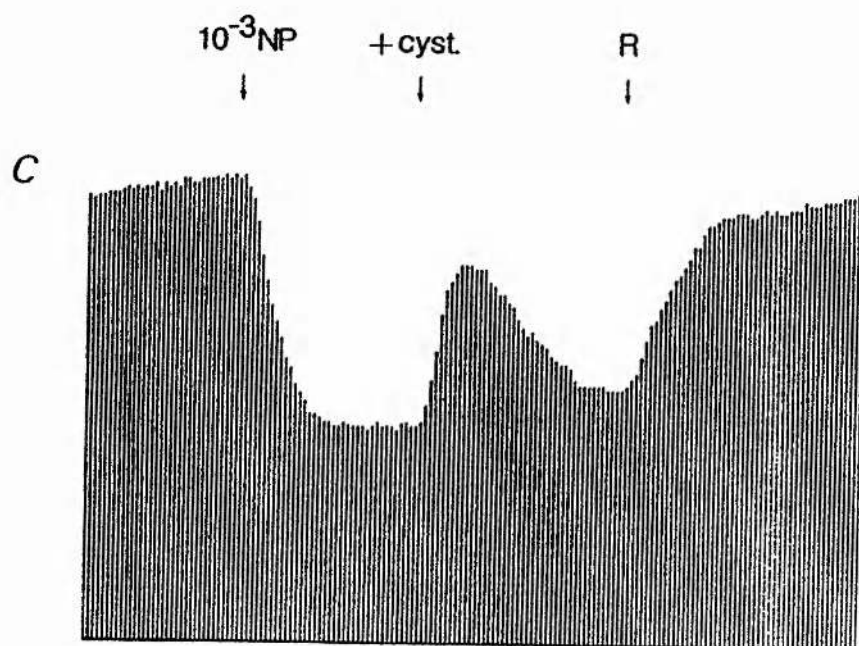
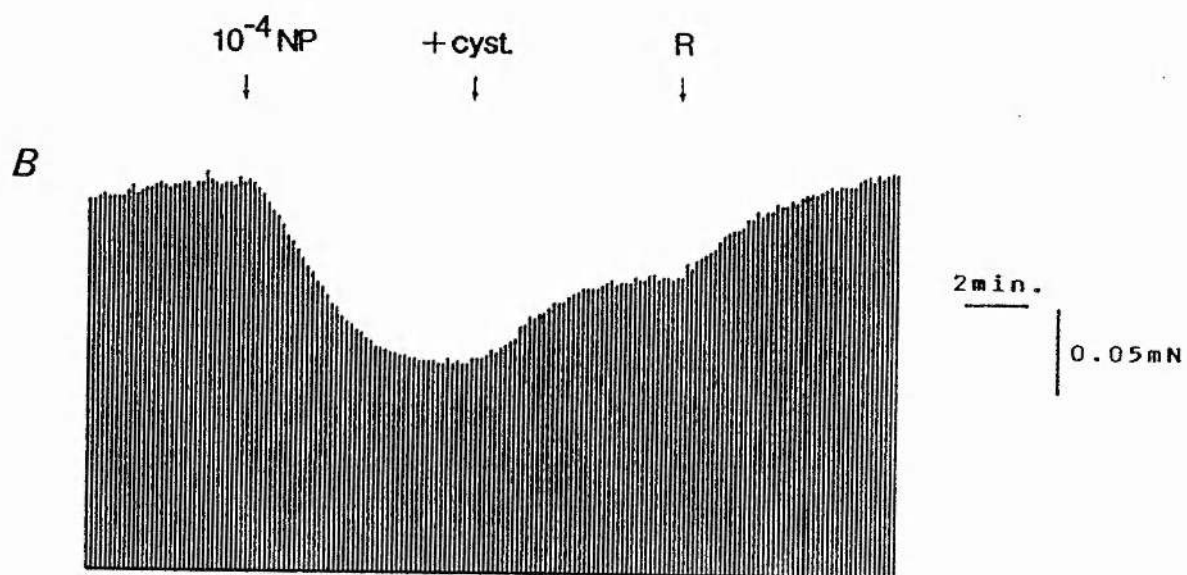
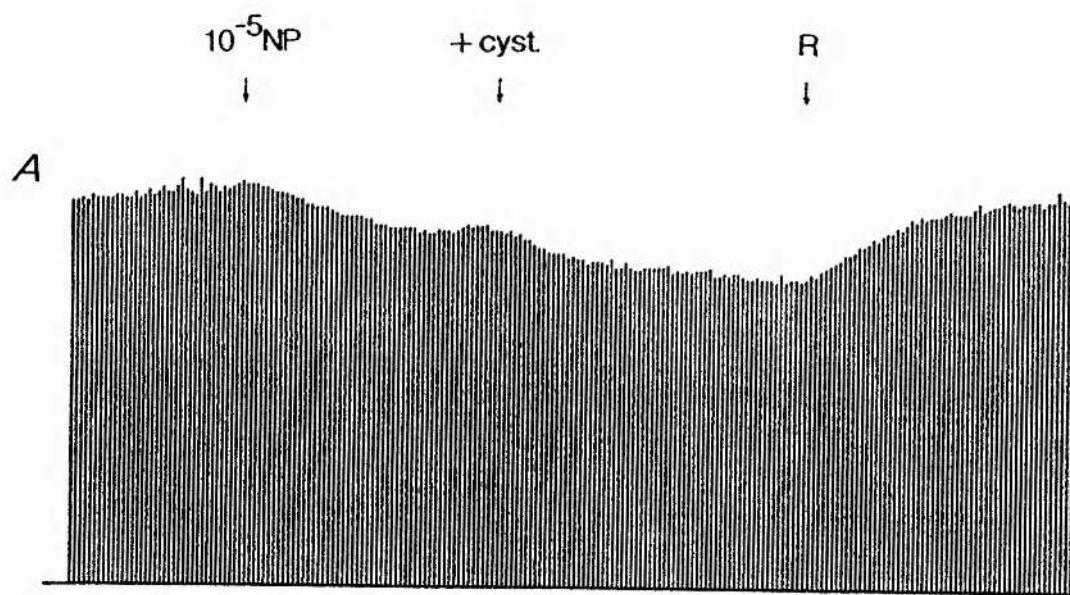
The effects of a standard (5mM) concentration were found to depend upon the concentration of NP used. Figure 7:3 shows the responses produced at three different NP concentrations. At the lowest [NP] ( $10^{-5}$ M) the addition of cysteine potentiated the response (A), increasing the extent of the depression by a factor of approx. 2 fold (ie. from 12% to 24%). At  $10^{-4}$ M NP (B) cysteine has the reverse effect: the initial twitch depression was reduced from 45% to only 24%. With  $10^{-3}$  NP (C), the recovery produced by cysteine was only transient: the depression was 53% initially, and temporarily recovered to 18%. However, continued perfusion caused a secondary depression to around 45% of control. Figure 7:4 shows a response to  $5 \cdot 10^{-3}$ M exposed NP, with and without cysteine. In the presence of cysteine the depression was reduced to 30%, as compared to 50% with NP alone.

The results of these experiments are summarised in figure 7:5. The dose response curve to photolysed NP alone has the expected sigmoidal shape, similar to that described previously for NP solutions exposed to 20mW of 488nm laser light ( Flitney & Kennovin, 1986). However, in the presence of cysteine, the log dose response curve takes on a bell-shape: at low [NP]'s the responses are augmented, but they become progressively reduced at concentrations less than  $2.5 \cdot 10^{-5}$ M NP. The shape of this curve is reminiscent of that obtained using NP solutions at high oxygen tensions (see fig.6:5). The reason why cysteine has this dual effect is not clear and some

Figure 7:3

This figure shows the effect of adding 5mM cysteine on the twitch depression produced by three different concentrations of NP exposed to 20mW 488nm laser light. The twitch depression induced by lower concentrations of photolysed NP are enhanced by the presence of cysteine (panel A) whereas it antagonises the response to higher NP concentrations (panel B & C). The reversal of the twitch depression caused by the addition of cysteine to  $10^{-3}$ M photolysed NP is transient. This is not seen at lower NP concentrations.





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### Figure 7:4

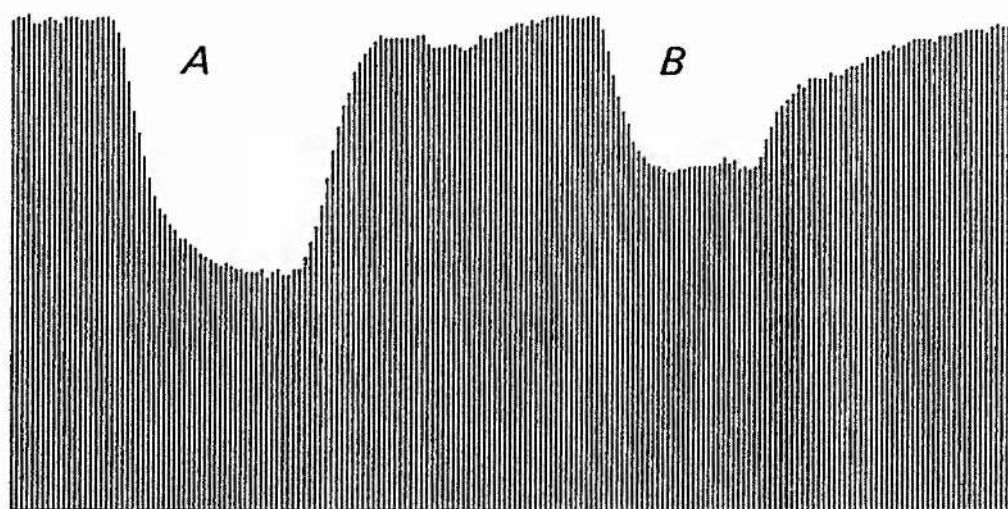
This figure shows the reduction in twitch tension produced by  $5 \cdot 10^{-3}$  M NP exposed to 20mw 488nm laser light in the absence (A) and presence (B) of 5 mM cysteine. It is clear that the presence of cysteine markedly reduces the size of the twitch depression ( from 57% to 30% ).

1

1

1

1



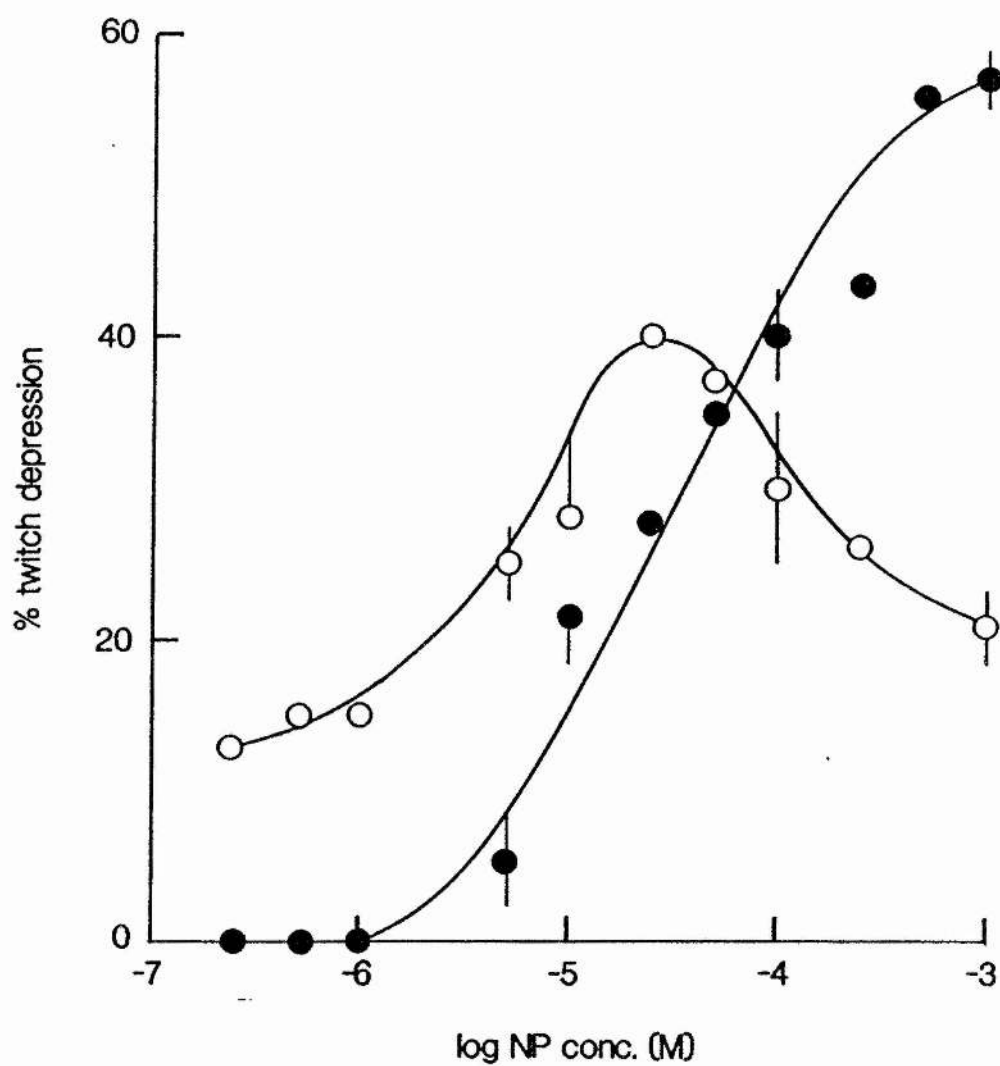
**.05 mN**

2min.

## Chapter 7

### Figure 7:5

The log dose response curve to photolysed NP in the absence (closed circles) and presence (open circles) of 5mM cysteine are shown in this diagram. Each point represents the mean (+ SE.) of 3-5 experiments. The presence of cysteine markedly alters both the shape and position of the dose response curve.



possibilities are considered later.

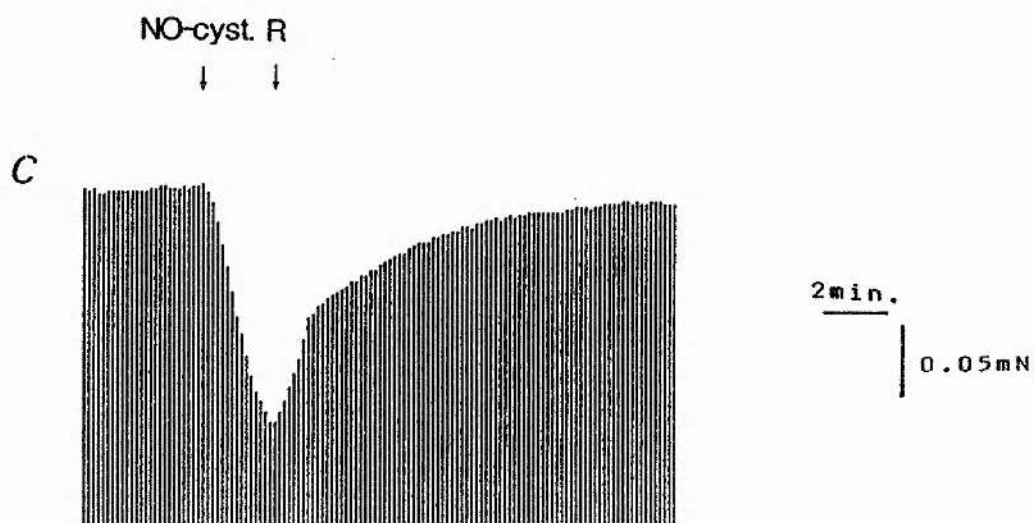
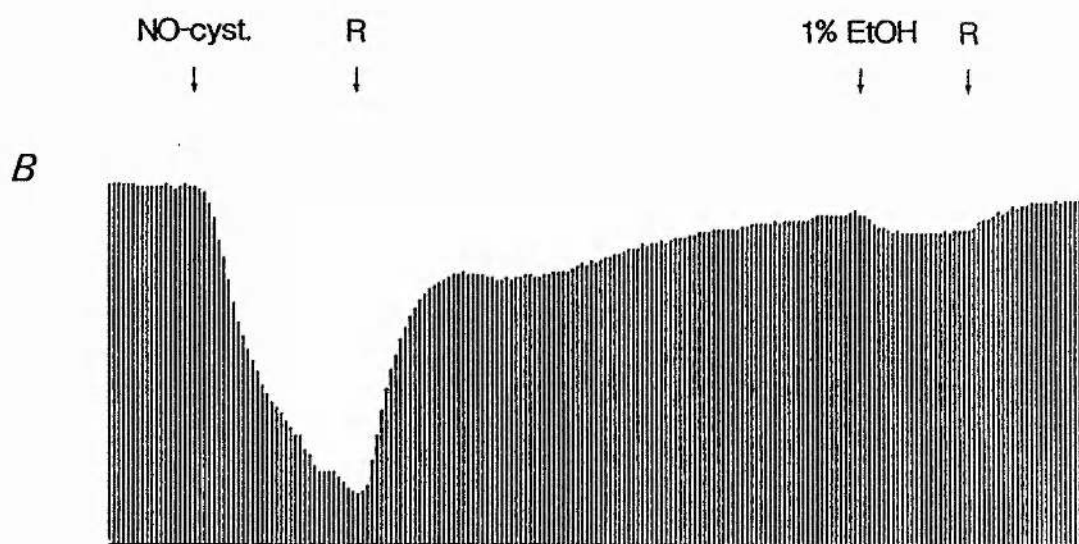
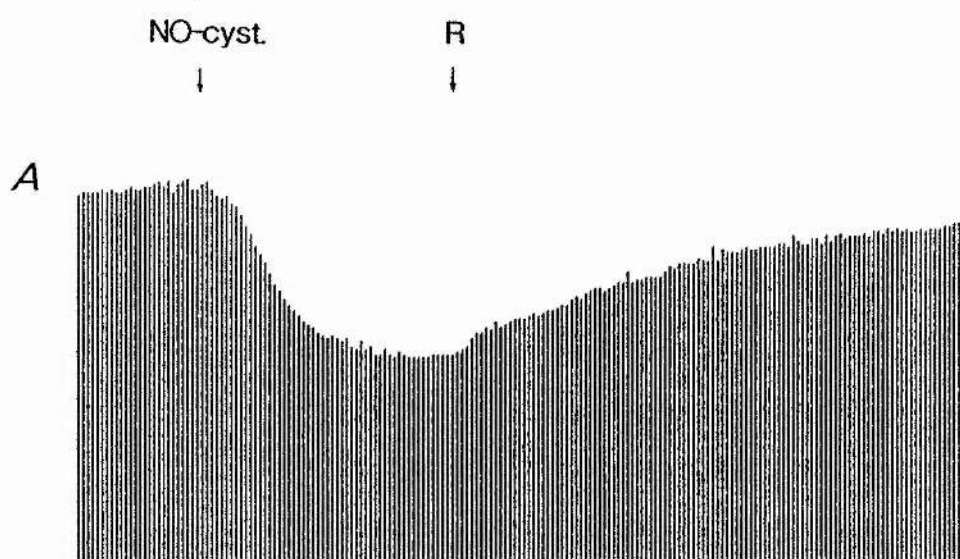
#### Effects of synthetic nitrosothiols on twitch tension

Nitrosothiols have been implicated in the stimulation of guanylate cyclase by NO and NP (Ignarro et al, 1981; Waldman & Murad, 1987). The effects of two nitrosothiols, nitrosocysteine and nitroso-N-acetyl-penicillamine, have therefore been tested. Nitrosocysteine was prepared and isolated in the crystalline form. It was then dissolved in ethanol before being diluted with Ringers solution to give a final concentration of  $10^{-3}$  or  $10^{-4}$ M. The purity of the sample used was not determined rigorously and so the concentrations quoted are only nominal values. Figure 7:6 shows the effects of  $10^{-4}$ M (panel A) and  $10^{-3}$ M (panel B) of freshly prepared nitrosocysteine. Panel C shows the effect of the same solution of  $10^{-3}$ M nitrosocysteine, approximately 1 hour after the response shown in figure panel B.

Freshly prepared nitrosocysteine has a profound negative inotropic response, reducing the twitch to 50% (A) or 10% (B) of its control value. The effect is rapid in onset, being essentially complete in 5 minutes. The same solution of nitrosocysteine as was used for the response illustrated in panel B, was applied again 1 hour later, when it was still effective, producing a 70% reduction of the twitch in approx. 2.5 mins. The last response (panel C) had to be curtailed as the volume of perfusate available was limited. The ethanol vehicle, tested without nitrosocysteine at the appropriate concentration has a negligible effect on the twitch (second response in panel B). The effects of nitrosocysteine were readily reversible on re-perfusing preparations with normal

Figure 7:6

This figure shows the effect of  $10^{-4}$ M (panel A) and  $10^{-3}$ M (panel B) preprepared nitroso-cysteine on the peak twitch tension of a frog trabeculum. This nitrosothiol has a strong negative inotropic action on frog ventricle, reducing the twitch to 44% (panel A) and 86% (panel B) respectively. Panel C shows that a subsequent application, 1 hour later of the same solution as in panel B, still produces a large twitch depression (70%). The effects of ethanol which is used as a solvent for the NO-cysteine, has a small but negligible effect on the twitch tension (panel B).





Ringers solution.

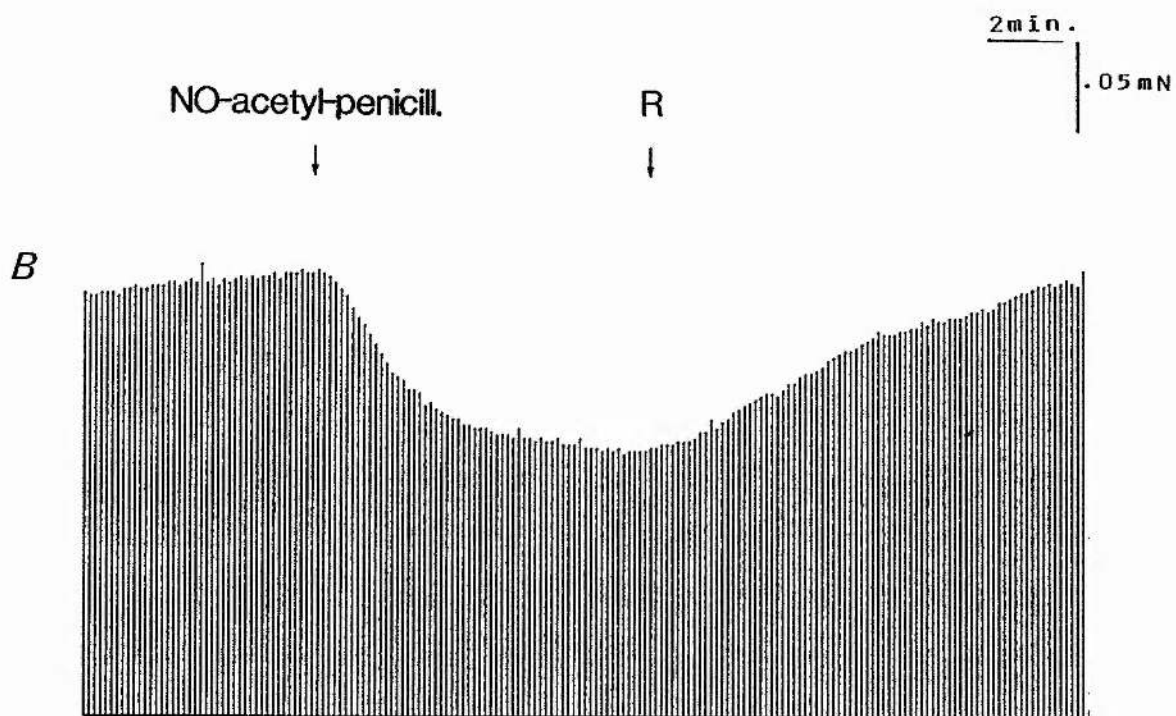
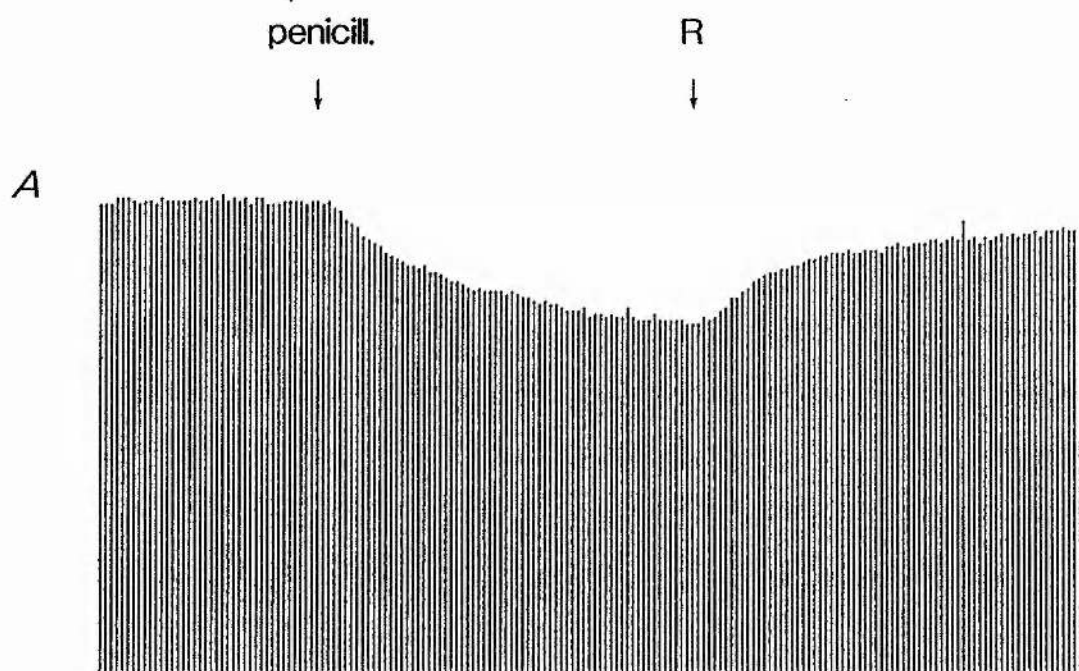
The apparent stability of nitrosocysteine in solution, as judged by its ability to significantly depress the twitch even after 1hr is puzzling. Nitrosocysteine is relatively unstable in solution with a half life of under 10mins (Craven & DeRubertis, 1983). Retention of the ability to suppress the twitch may therefore mean that NO liberation could be responsible for its effects. This last possibility has not been tested. The question might be answered by studying the responses in the presence and absence of oxygen or haemoglobin.

Responses elicited by nitrosopenicillamine are illustrated in figure 7:7. This compound is relatively stable in solution. The parent compound, acetylpenicillamine, was also tested. This was found to have a significant depressent effect, but like that produced by cysteine, it was more effective on preparations that had already been subjected to earlier treatments and did not significantly affect fresh preparations. However, the nitroso derivative had a powerful negative inotropic effect. Figure 7:7 (B) shows that 50uM nitroso-acetylpenicillamine produced a 41% depression of the twitch. The effect like that of nitrosocysteine, was fully reversible on perfusing the preparation with normal Ringers solution. There was insufficient material available to allow an adequate study of the relative efficacies of nitrosocysteine and nitroso-acetylpenicillamine.

Figure 7:7

Panel A shows the effect of 0.1mM acetylpenicillamine alone, which was applied at the end of an experiment and has a similar action to cysteine in reducing twitch tension. The depression of the twitch does not however spontaneously recover in acetylpenicillamine solution.

Panel B shows the effect of  $5 \cdot 10^{-5} \text{M}$  nitroso-acetyl-penicillamine (NOaP) on the frog ventricular peak twitch tension. Even at this low concentration, NOaP markedly reduces the twitch tension (41% twitch reduction).



Discussion

The results described here show that cysteine alone does not affect the twitch contraction of trabeculae that have not previously been subjected to drug treatment. After procedures which depress the twitch, cysteine can then evoke a transient reduction in peak twitch tension, the size of which appears to be related to the extent of the depression caused by prior drug treatment. This suggests that the inotropic ability of cysteine is more dependent upon the legacy from previous responses than upon any inherent capacity to alter the twitch. There can be little doubt that the ultimate site of action of NP, and presumably the other synthetic nitrosothiols too, is on the enzyme guanylate cyclase and this raises the possibility that prior exposure to agents which depress the twitch may leave the enzyme in a refractory condition. It has been suggested that the nitrovasodilators mediate their effects on vascular smooth muscle by oxidising thiol groups to disulphides (Needleman, Jaschik & Johnson, 1973). The activity of purified GC in vitro has also been shown to be markedly affected by the availability of SH groups (Waldman, Rapoport & Murad, 1987). This may also be true of the GC in frog ventricular cells, since the evidence of the present study suggests that NP acts through NO activation of GC. If we assume that this is so, then treatment with photolysed NP could result in the conversion of some SH groups to disulphides, causing activation of the enzyme and consequent depression of the twitch. There would then be fewer SH groups free to respond to another NP challenge. This is

thought to be the basis of the tolerance shown by vascular smooth muscle to repeated exposure to the nitrovasodilators (Needleman & Johnson, 1973). This has recently been shown to occur at the level of the purified GC enzyme (Waldman, Rapoport & Murad, 1987). Cysteine is a potent sulphhydryl reducing agent, able to re-convert disulphide bridges back to free thiols. This could lead to stimulation of basal enzyme activity, similar to that seen following treatment of crude arterial supernatants with dithiothreitol (DTT). In fact DTT has been reported to activate, to have no effect on, or else to inhibit crudely purified GC, depending upon whether the GC is initially in the oxidised, in a 'ground state' or is reduced, respectively (Waldman & Murad, 1987). If the guanylate cyclase in 'fresh' trabeculae were in a 'ground' oxidation state then cysteine would not affect the basal rate, and so would not alter twitch tension. Following oxidation (and activation) of the enzyme (eg. by photolysed NP or by nitrosothiols), the enzyme would remain in the oxidised state, such that subsequent reduction by cysteine could then cause re-activation. It may seem strange that oxidised enzyme does not remain activated but this has been shown to be the case with purified guanylate cyclase (Braughler, Mittal & Murad, 1979). Brandwien & Murad (1983) showed that the activity of GC following stimulation with nitric oxide decayed with time, despite the continued presence of nitric oxide throughout. The rate of decrease was found to be temperature dependent and could be prevented by addition of DTT to the incubation medium. Once the activity had spontaneously decreased to basal rate, the addition of DTT increased the activity back to its

maximal level (Brandwien & Murad, 1983).

These considerations suggest that the variable inotropic actions of cysteine may be a consequence of the oxidation state of guanylate cyclase. This could account for the spontaneous recovery of the twitch in the presence of cysteine, because the enzyme would become progressively more reduced back to its neutral 'ground' state. Further applications of cysteine would then be ineffective which is in agreement with the observed results.

The lack of an effect of unphotolysed nitroprusside in the presence of cysteine suggests that cysteine is unable to cleave the nitrosyl moiety, contrary to previous reports (Ignarro et al, 1981). The reported formation of nitrosocysteine under similar conditions may be an artefact, caused by the release of NO through photolysis, which then combines with cysteine (Ignarro et al, 1981). Other authors agree that mixtures of NP and cysteine at pH 7.6 do not form nitrosocysteine (Craven & DeRubertis, 1983). Cysteine and/or other thiols may normally be present in frog ventricle but they do not seem to be involved in nitroprusside cleavage. Whether this is true for other nitrovasodilators is not known. The more permeant GTN, for instance may elicit an effect on frog ventricle due to degradation by intracellular thiols, but further investigation is required.

The effects of photolysed NP are markedly modified by the addition of cysteine. The free nitric oxide produced by photolysis of NP would be expected to react with cysteine in solution to form nitrosocysteine. This is in agreement with the reported ability of cysteine to react with either free



nitric oxide or photolysed NP to form nitrosocysteine (Pryor, Church, Govindan & Crank, 1982; Ignarro et al, 1981). Since nitrosocysteine is reported to be more potent at stimulating guanylate cyclase than free nitric oxide, it was anticipated that cysteine would potentiate responses to photolysed nitroprusside (Ignarro & Greutter, 1980). The direct reducing action of cysteine should assist in this action, as reducing agents have been shown to potentiate the activation of crude and purified soluble guanylate cyclase by nitrovasodilators and by nitric oxide (Kimura, Mittal & Murad, 1975). A potentiating effect was seen for lower concentrations of nitroprusside. Concentrations of NP which were too weak to elicit a response alone, markedly reduced the twitch in the presence of cysteine. Above concentrations of  $2.5 \times 10^{-5} \text{ M}$  NP however, cysteine antagonised the response, and the extent of this effect increased with increasing NP concentrations. Instead of shifting the NP dose response curve leftward and upward, cysteine changed the shape of the curve, from its normal sigmoidal to a bell shape. The reason for this is not clear, as any effect of cysteine on free nitric oxide (i.e. scavenging NO to form NO-cysteine and so impeding its diffusion into the cell) could occur throughout the range of nitroprusside concentrations and so would not explain the change in shape of the dose response curve. The similarity of this curve to that obtained at high oxygen tensions might suggest a similar mechanism of action. It was postulated that the latter could be due to the combined effects of a high oxidising environment, created by using oxygen saturated Ringer, together with high nitric oxide concentrations, to

## Chapter 7

'overoxidise' guanylate cyclase and result in decreased activity. The reducing environment produced by cysteine could have similar effects in moving the redox state of the enzyme away from its optimal value. It has been shown that cysteine and DTT reduce the activity of guanylate cyclase (activated by the oxidant dehydroascorbic acid) in both intact cells and broken cell homogenates by a factor of 2 (Haddox, Stephenson, Moser, Goldberg, 1978). The reason why increasing the oxidant (ie. nitric oxide) concentration while keeping that of the reductant (ie. cysteine) constant, decreases the activity of the enzyme is unclear. The postulated role of different critical sulphydryl groups being responsible for different aspects of the enzyme activation (ie. basal activity, nitric oxide activation, oxygen inactivation etc) and the suggestion that each is separately susceptible to various oxidising and reducing agents, gives some idea of the complexity of the regulation of guanylate cyclase by redox agents (Waldman & Murad, 1987). Another complication is that most of these studies are performed on the isolated enzyme which could be in different redox states depending on the isolation procedures used, the previous oxidation state in the tissue, and the particular environment of the enzyme incubation. For these reasons it is hard to draw any conclusions about the in vivo situation from in vitro studies.

Preprepared nitrosocysteine was shown to be a potent negative inotropic agent that quickly depresses the twitch (90% in under 5min). This may be due to the relatively high concentration of nitrosothiol used (1mM) which is approximately 20 times greater than that which could be



## Chapter 7

produced by reacting cysteine with the highest concentration of photolysed NP used in these experiments. Nitrosocysteine is a very unstable compound which decomposes even in the crystalline state and has been shown to rapidly decompose in solution of pH 7.6 with a half life of 10 minutes (Craven & DeRubertis, 1973). It was thought therefore that nitrosocysteine would lose some of its potency with time, but this was not the case, as even after an hour the same solution of nitrosocysteine could elicit an equivalent depression of the twitch. Indeed, the later application appeared to depress the twitch at a faster rate than the initial one. This raises the question whether the intact nitrosocysteine molecule is responsible for these effects or whether they are due instead to one of its decomposition products. Since decomposition of NO-cysteine would liberate nitric oxide then this could directly reduce the twitch. Previous studies have reported that a solution of fully decomposed nitrosocysteine is equally as effective as a freshly prepared one at stimulating guanylate cyclase activity (Craven & DeRubertis, 1983). Evidence that nitroso-acetyl-penicillamine which is more stable than NO-cysteine, still elicits a negative inotropic response suggests that intact nitrosothiols are effective at stimulating GC. Whether nitroso-acetyl-penicillamine is as effective as NO-cysteine cannot be ascertained at the present time, but it would seem that both intact or decomposed nitrosothiols may be potent negative inotropic agents.

## CHAPTER 8

The effects of photolysed and intact nitroprusside on  
mammalian vascular smooth muscle.

Introduction

The preceding chapters have shown that the action of NP on frog ventricle is dependent on prior photolysis of the molecule to release nitric oxide. As NP is used clinically as a vasodilator, it seemed appropriate to investigate whether this mechanism played a role in its effects on vascular smooth muscle. Numerous studies have shown that NP elicits a strong relaxant effect on precontracted isolated smooth muscle preparations, but little if any have appreciated the photosensitivity of the drug. If NP was not protected from light in these studies then little is known about whether photolysis has a role in eliciting the drug action in smooth muscle. When intravenously infused into whole animals, including man, photolysis of NP in the infusion bottle could affect the drug action. Indeed prior photolysis has been suggested to be responsible for the release of cyanide upon infusion into whole animals (Bisset, Butler, & Glidewell, 1981). As has already been said, NP along with the other nitrovasodilators are postulated to act through release of nitric oxide to form nitric oxide containing intermediates. This is thought to involve thiols, especially cysteine, associated with the smooth muscle, which cleave the NO off the parent molecule. Some groups have erroneously postulated that NP spontaneously forms nitrosocysteine in the presence of cysteine, but this can be attributed to photolytic release of nitric oxide which then reacts with the thiol (Ignarro et al, 1981; Craven & DeRubertis, 1978). Another

## Chapter 8

group aware of the photosensitivity of NP, have shown that the nitrosyl group is cleaved off NP in the dark by arterial soluble fraction (Leewenkamp et al, 1981). Since it is the liberation of NO that is responsible for the drug action, then this would suggest that intact NP can elicit an effect on smooth muscle, although photolytic liberation of NO may potentiate this effect.

The experiments about to be described investigated whether prior photolysis of NP affects the relaxant response in vascular smooth muscle. As was expected, the results show that photolysis augments the relaxant effect which lends further evidence to the hypothesis that release of the nitric oxide is involved in mediating smooth muscle relaxation.

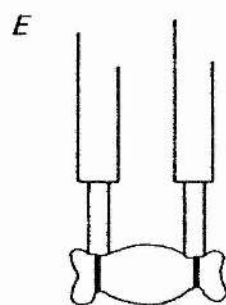
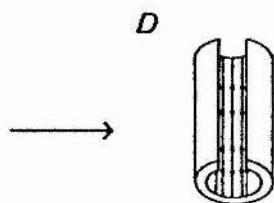
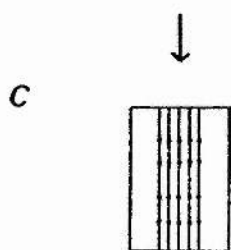
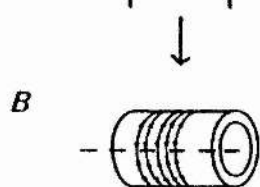
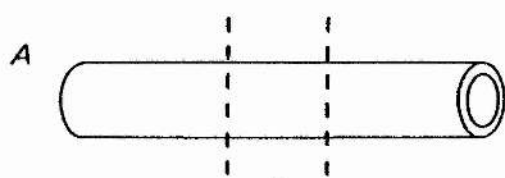
Methods

**Dissection:** Experiments were made using small strips of isolated rabbit ear artery. A length of artery (2-3 cm) was dissected from the dorsal surface of a rabbit's ear and placed in a dissection dish filled with Kreb's solution (composition (mM): NaCl, 118; KCl, 4.7;  $\text{NaHCO}_3$ , 25;  $\text{NaH}_2\text{PO}_4$ , 1.15;  $\text{CaCl}_2$ , 2.5;  $\text{MgCl}_2$ , 1.13; glucose, 5.6; bubbled with 95%  $\text{O}_2$ :5%  $\text{CO}_2$ ; pH 7.4). A segment of artery was cut out, rolled and snared with tungsten wire loops, such that the smooth muscle fibres were orientated in the direction of motion of the force transducer (fig. 8:1). This arrangement ensured that the luminal surface of the artery was exposed directly to the bathing solution. In some experiments a more sensitive force transducer was employed which allowed small strips of unrolled artery to be used.

Arterial strips were mounted in the apparatus and left to equilibrate for 1-2 hrs in Kreb's solution. The optimum length for contractures was then established as follows. Isometric contractures were elicited by exposing preparations to 5uM phenylephrine (PE), a relatively stable  $\alpha$ -agonist. This was repeated several times, increasing the length of the preparation (0.1 mm increments) between successive contractures, until the maximum contracture tension was obtained (fig. 8:2). Preparations set up in this way performed consistently throughout the experiments, as can be judged from the last two contractures (see fig 8:2, 4&5), taken at the beginning and at the end of an experiment.

Figure 8:1

This diagram shows the dissection of the rabbit ear arterial preparation. A small segment of isolated rabbit ear artery is cut away as shown (A). A longitudinal incision is made along one side (B) and the artery opened out to form a flat sheet of tissue (C). This is then rolled in a direction  $90^{\circ}$  to the original curvature (D) and snared in the tungsten wire loops of the muscle holders (E). This procedure ensures that the long axis of the smooth muscle fibres (indicated by the arrowed lines) is parallel to the direction of motion of the force transducer.



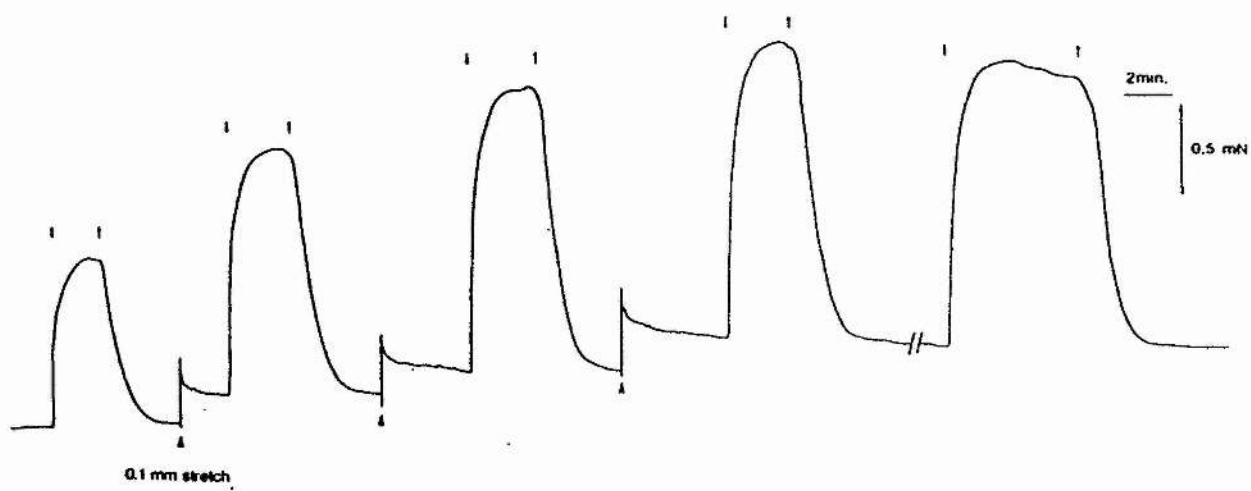
1.0 mm



Figure 8:2

This diagram shows a tracing from an original chart recording illustrating the procedure used to standardise the length of the muscle. The preparation was stretched by 0.1mm (large arrows) between successive contractures elicited by a standard dose ( 5  $\mu$ M) of phenylephrine (downward arrows), until the size of the contracture was maximal. The contracture then remained constant throughout the duration of the experiment, as demonstrated by the last two contractures on the right, as the far right one was recorded at the end of the experiment.





**Apparatus:** Contractions were elicited by exposing preparations to PE, instead of using electrical stimulation as in the experiments with frog trabeculae. This required a minimum of two inlet routes to the muscle chamber: route A, the normal one incorporating the exposure tube; and a second route B, in which solutions were made to flow through a parallel tube, optically isolated from, but close to, the exposure tube (fig. 8:3). Solutions passing through the exposure tube (route A) were selected in the usual way by means of the six-way tap. Kreb's solution flowed continuously through route B. Each tube was connected to one inlet of a two-way constant flow tap (T). This permitted only one of the two solutions to reach the muscle chamber, while the other flowed to waste. The flow rate through each route was adjusted to be the same (10mls/min.). The combined volume of the muscle chamber and its inlet pipe was kept small (0.1ml) to allow rapid solution changes and to minimise 'dead space' effects. Standard contractures were elicited by switching from normal Kreb's solution (route B) to Kreb's solution containing PE (route A; through exposure tube). The duration of each contracture was determined either manually, or by using a microcomputer which operated a mechanical drive to the two-way tap.

All solutions were prepared and kept in the dark prior to use, unless stated otherwise.

It was necessary to maintain the preparation at 35°C throughout the experiment. This was achieved by means of a heat exchanger system (fig.8:3). A peristaltic pump (P) was

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### Figure 8:3

Apparatus used to maintain the temperature of the solution at 35°C

C - heat exchange coil (in cross section to show pipes within)

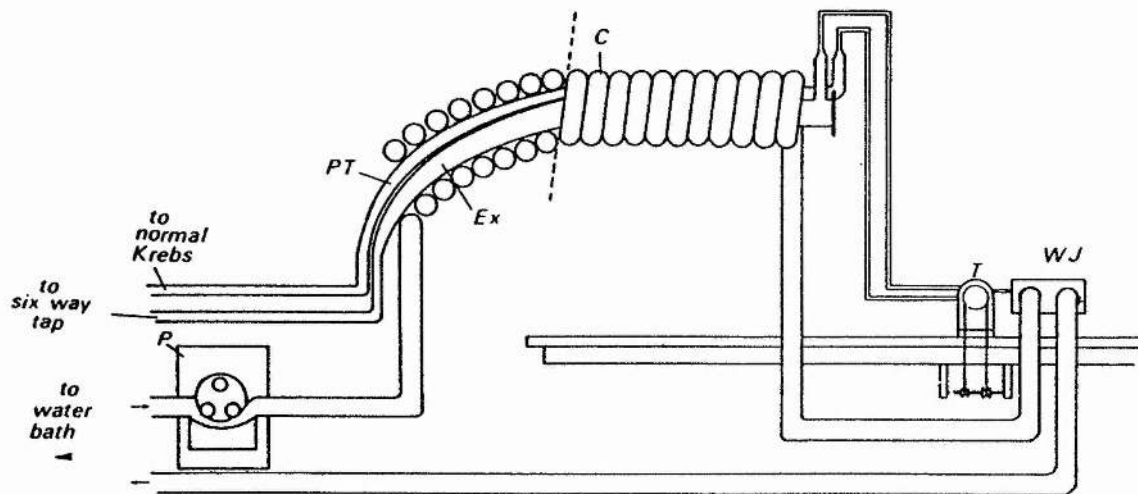
Ex - exposure tube (route A)

PT - perfusate tube (route B)

T - motorised two-way constant flow tap

WJ - water jacket around muscle chamber

P - peristaltic pump to circulate warm water



made to circulate warm water through a coil (C), wrapped tightly around the inlet tubes, and then on through a water jacket (WJ) surrounding the muscle chamber. The preparation was maintained at the required temperature ( $+ 1^{\circ}\text{C}$ ) by careful adjustment of the temperature of the fluid entering the coil; the rate of circulation of water through the coil; and the flow rate of experimental solutions through the muscle chamber.

**Experimental Protocol:** Two types of experiments were performed.

Type 1 experiments: ability of NP to attenuate PE contractures.

Two procedures were used;

A. Responses to different concentrations of PE were recorded and the results obtained used to construct a 'standard' dose response curve. The experiment was then repeated but with PE solutions containing  $5\mu\text{M}$  NP that had been either kept in the dark, or else subjected to photolysis by: (i). pre-exposing solutions to 'white' light, as described in chapter 3, or (ii). illuminating solutions in the exposure tube, 'en route' to the muscle chamber, with  $20\text{mW}$  laser light at a wavelength of  $457.9\text{nm}$ .

B. Groups of three contractures were elicited as follows: the first, to  $1\mu\text{M}$  PE alone; the second, to  $1\mu\text{M}$  PE + protected NP; and the third, to  $1\mu\text{M}$  PE + laser exposed ( $20\text{mW}; 457.9\text{nm}$ ) NP. The concentration of NP used (range:  $25\text{nM}$ - $500\mu\text{M}$ ) was changed between each set of three

contractures.

Type II experiments: The ability of NP to relax pre-contracted arterial strips.

The ability of protected NP and of laser photolysed NP (20mW, 457.9nm) to cause relaxation of pre-contracted arterial strips was studied. Again, two procedures were used:

A. PE contractures were evoked in the usual way. When the steady plateau of tension had been reached (generally, 1-2min.) the perfusate was switched from PE alone to PE + 5uM unexposed NP (route A). After 1-2min. the laser was switched on for a period of 1-2min. It was then switched off so that the preparation was once again perfused with PE + unexposed NP. Then, generally 4-5min. later, the perfusate was switched to normal Kreb's solution (route B) and the preparation allowed to relax.

B. Here, the relaxant effect of different concentrations of NP (range: 1nM-25uM) was tested on preparations precontracted with a standard dose (1uM) of PE. The efficiency of unexposed and laser photolysed NP was then compared.

### Results

The results of these experiments show that NP is able to attenuate contractures to PE and that it can also cause relaxation of pre-contracted preparations. In both cases, its efficacy is potentiated by photolysis.

The ability of NP to attenuate PE contractures

Type 1A experiments: Effects of a fixed dose (5uM) of NP on PE contractures. Figure 8:4 shows a series of superimposed contractures produced by PE alone (top traces), PE+5uM protected NP (middle traces) and PE+5uM pre-exposed ('white' light) NP (bottom traces). The concentrations of PE used are given beneath each set of contractures.

These recordings show that both intact and photolysed NP can depress PE contractures. The results of 5 similar experiments are summarised in the dose response curves of figure 8:5. The peak tension generated is here expressed as a percentage of the maximum produced by PE alone (open squares). There are two points to note. First, intact NP causes the dose response curve to be displaced to the right (filled circles) shifting the  $ED_{50}$  value for PE from 0.73uM to 2.0uM. Secondly, pre-exposing NP solution to white light significantly enhances its depressant effect, causing the dose response curve to be shifted even further to the right (open circles) such that the  $ED_{50}$  value is now increased from 0.73uM to 3.3uM. Quantitatively, photolysis of NP under the experimental conditions used here increased its potency by  $(3.3/2.0 =) 1.65$  fold.

The potentiating effect of photolysis is significantly greater when NP solutions are exposed to laser

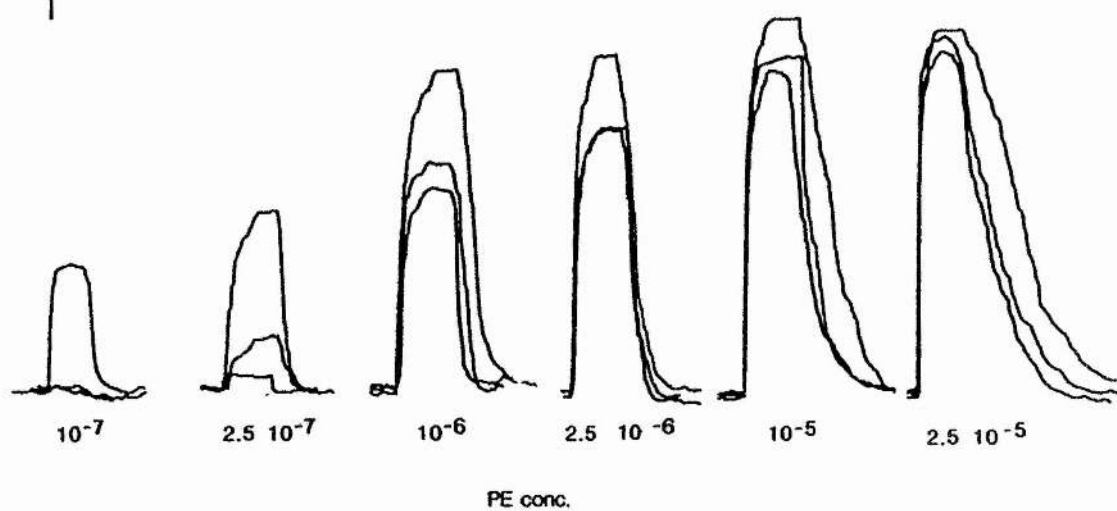
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### Figure 8:4

Contractures produced by phenylephrine in the presence of 5  $\mu$ M unexposed NP (middle traces), or 5 $\mu$ M photolysed NP by pre-exposure to white light (bottom traces), are shown superimposed on the control contracture to PE alone (top traces). The concentration of PE used is shown below each set of contractures.



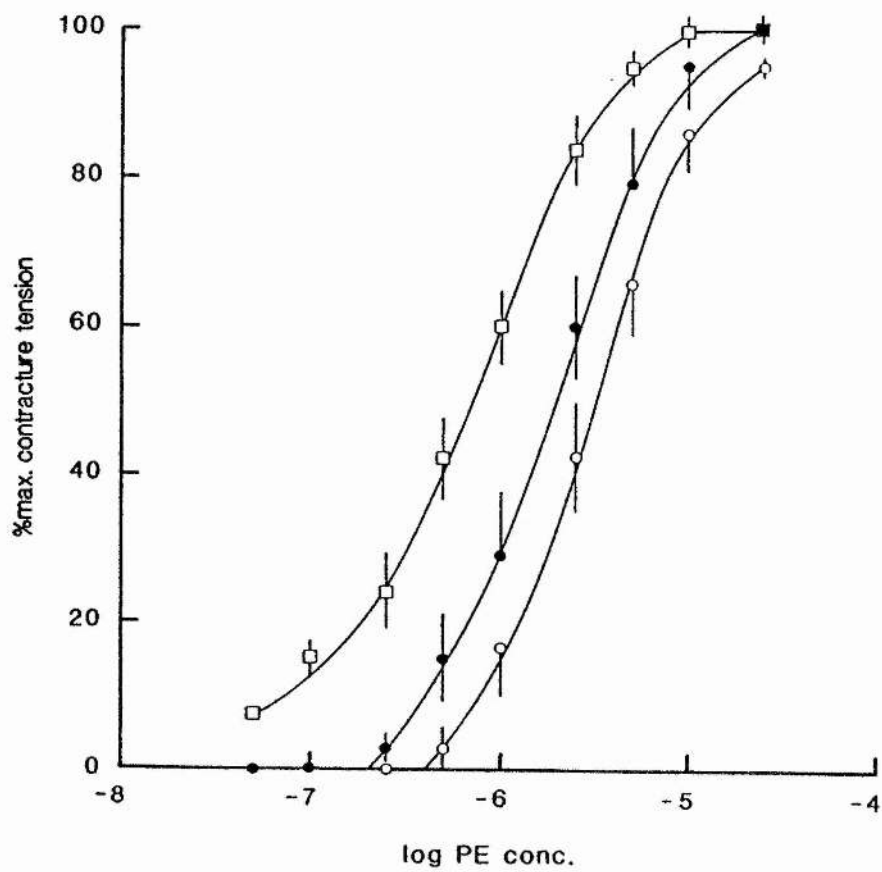
0.5mN  
2min



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### Figure 8:5

Plotted here are the log dose response curves to PE alone (white squares) and in the presence of 5uM nitroprusside that was either protected from (black dots) or photolysed by (white dots) exposure to white light. Each point is the mean value from 5 experiments (+ standard error).



light en route to the muscle chamber. Figure 8:6 shows a series of contractures to PE alone (upper traces), PE containing 5uM protected NP (middle traces) and to PE containing 5uM laser exposed NP (bottom traces). Except for the illumination conditions, these recordings can be compared directly to those shown in figure 8:4. Again, it is clear that protected NP depresses PE contractures. The effect is more pronounced at the lower PE concentrations. However, notice that the depression caused by laser photolysis is significantly greater than that seen with solutions pre-exposed to white light. This is illustrated by comparing the contractures produced by 0.25uM PE as preparations were able to generate an appreciable force in the presence of solutions pre-exposed to white light, but laser photolysis abolished the contracture completely. This point is taken up again later.

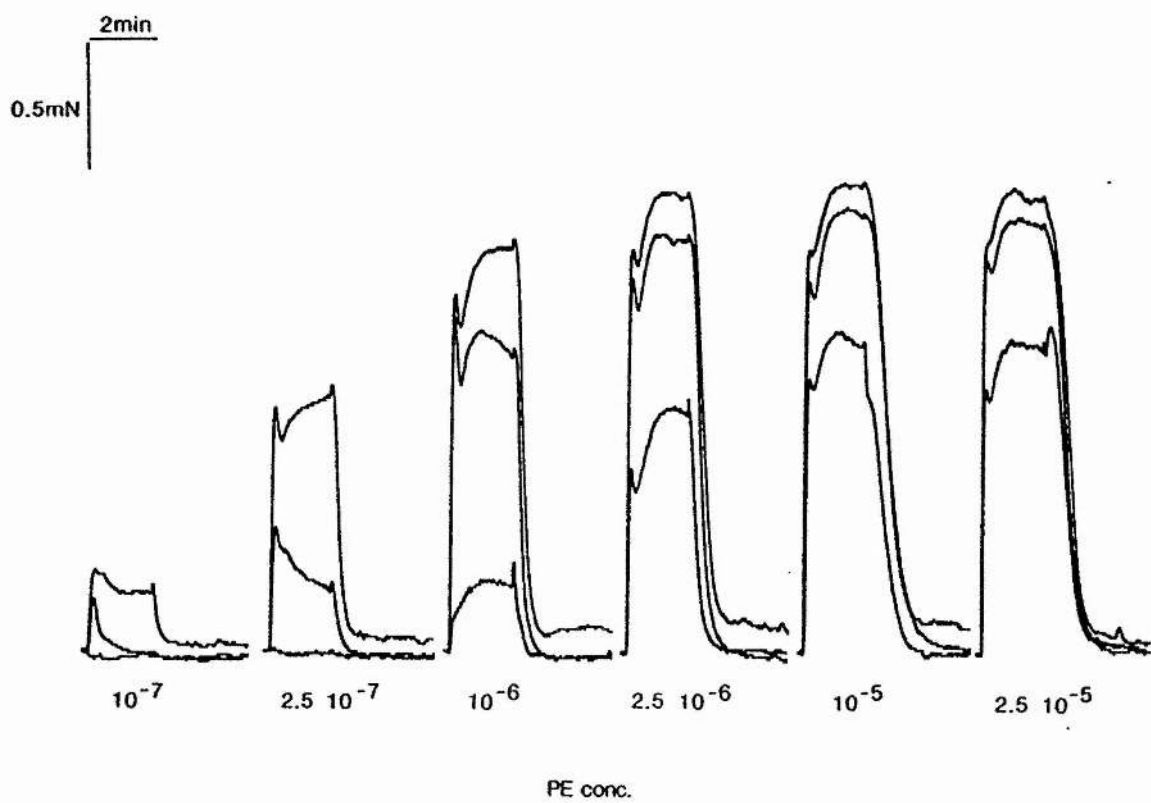
Type 1B experiments: Effect of different concentrations of NP on contractures evoked by a constant dose (1uM) of PE. Figure 8:7 shows two sets of paired contractures, elicited by 1uM PE alone and 1uM PE in the presence of differing concentrations of NP (range:  $5 \cdot 10^{-7}$  -  $10^{-4}$  M). The upper row demonstrate the effect of intact NP and the lower row the effect of NP pre-exposed to white light.

There are two points to emphasise concerning these data. First, the attenuation of the PE contracture increases with increasing NP concentrations. This is true for both intact and photolysed NP. Secondly, the degree of the depression of the contracture is invariably greater after

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### Figure 8:6

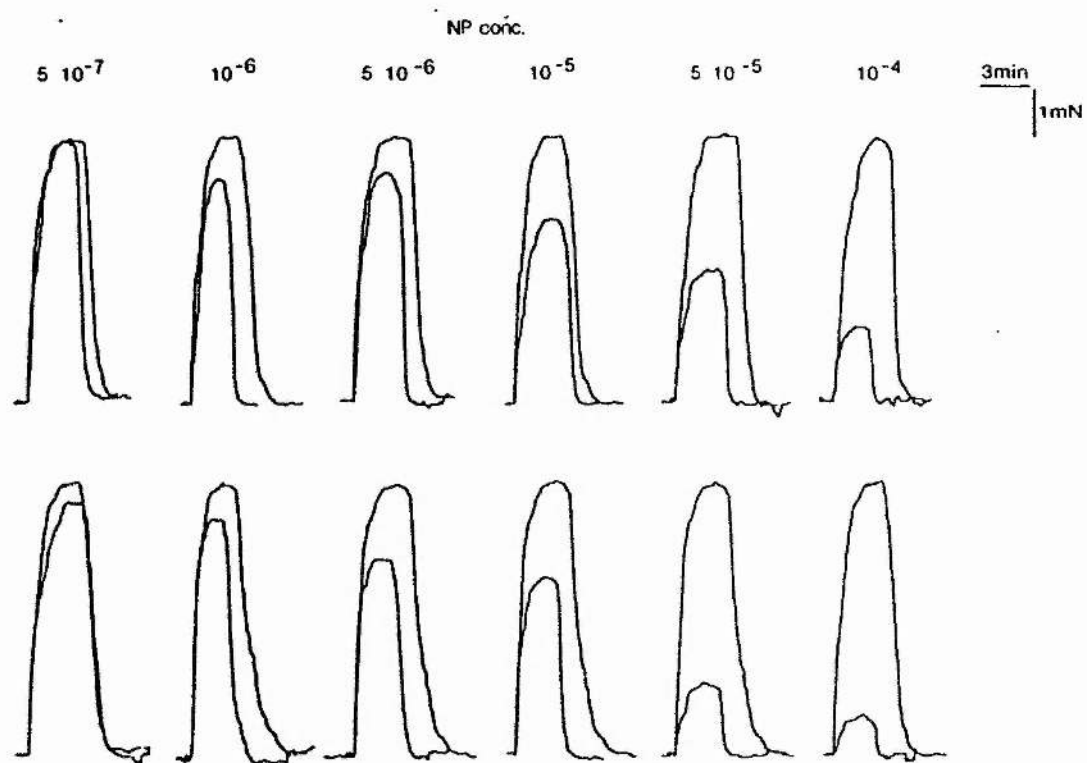
Contractures to various concentrations of phenylephrine in the presence of nitroprusside that was either unexposed (middle traces) or photolysed by 20mW 457nm laser light (bottom traces) are shown superimposed on the preceding control contracture to PE alone (top traces).



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### Figure 8:7

This diagram shows the antagonism produced by various doses of nitroprusside on contractures produced by  $10^{-6}$ M phenylephrine. The PE contractures produced in the presence of intact (upper panel) and photolysed (by white light; lower panel) NP are shown superimposed on the preceding control contracture to PE alone (top traces). The nitroprusside concentration used is shown above each group of contractures.

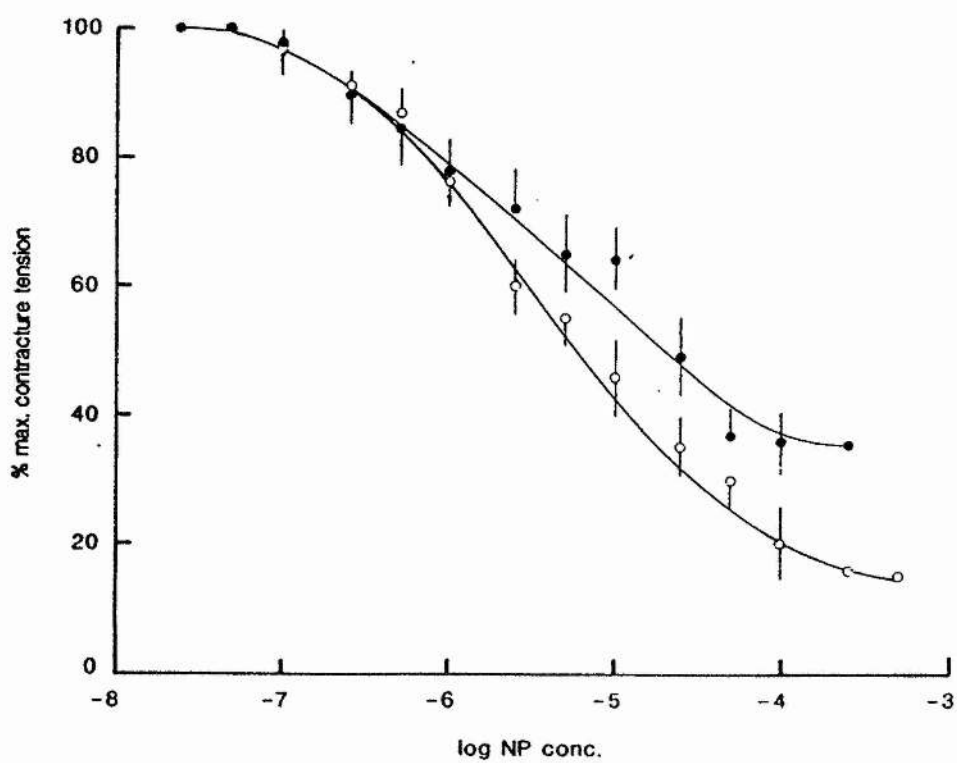




## Chapter 8

### Figure 8:8

The antagonistic log dose response curves for nitroprusside, that was either completely protected from light (closed circles) or preexposed to white light (open circles), on the contracture produced by  $1\mu\text{M}$  phenylephrine are shown.



photolysis.

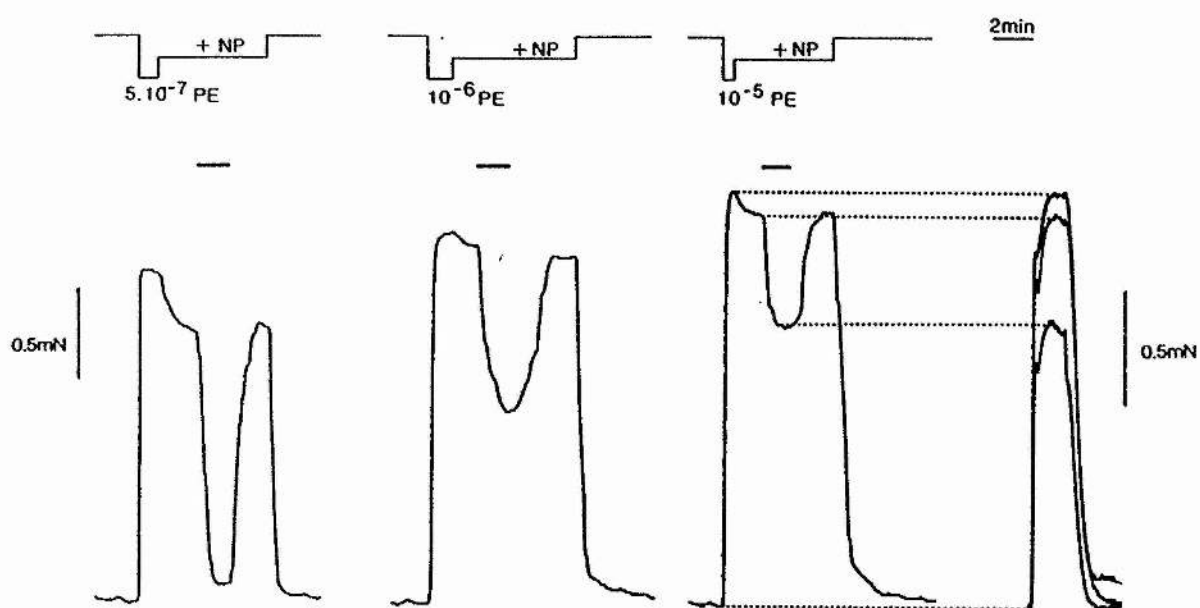
The accumulated results from 9 experiments of this type are plotted in figure 8:8. Here, the maximum contracture tensions recorded in the presence of protected (filled circles) and pre-exposed (open circles) NP are expressed as a percentage of those obtained with PE alone. There is little difference between the two sets of data for [NP]'s  $< 1\mu\text{M}$ . However, above  $1\mu\text{M}$  the curves diverge and the contracture tension falls steeply following exposure of NP to white light. The concentrations required to produce a 50% reduction in contracture tension are  $5.4\mu\text{M}$  for pre-exposed NP, as compared with  $20.3\mu\text{M}$  for intact NP.

#### Relaxant effects of NP on pre-contracted arterial strips

Type 2A experiments: Effects of a fixed dose of NP ( $5\mu\text{M}$ ) on strips precontracted with PE. The ability of protected and laser exposed NP to relax pre-contracted preparations was studied. Figure 8:9 shows three recordings which illustrate the experimental procedure. The upper line shows the pattern of solution changes. Arterial strips were first contracted by perfusing them with Kreb's solution containing PE alone. The perfusing solution was then changed during the plateau phase of the contracture, to one containing the same concentration of PE plus  $5\mu\text{M}$  protected NP. This caused preparations to slowly relax to a new steady level of tension. The laser was then switched on for approximately 1 minute (horizontal bars). The effect was immediate; photolysis of NP resulted in a dramatic, fast relaxation, causing the tension to fall to a much lower steady state

Figure 8:9

Preparations were precontracted by various concentrations of phenylephrine and then perfused with protected nitroprusside. This causes the preparation to relax. After the force had reached its new steady level, the perfusate was illuminated by 20mw 457nm laser light. This causes an increase in the extent of relaxation. The changeover of solutions is shown by deflections of the line in the top panel and the duration of illumination depicted by the thicker horizontal bar. The superimposed individual contractures (on the right) are similar to those in figure 8:8 for  $10^{-5}$  M PE, plotted on the same timescale, to show the similar effects of NP using the two different methods.



level. The magnitude of the 'extra' relaxation upon photolysis varied inversely with the PE concentration; thus, at 0.5 $\mu$ M PE, the contracture is virtually abolished during the period of illumination, while at 10 $\mu$ M PE, the force stabilises at about 75% of its initial (PE alone) control value. The recovery of tension, when illumination ceases, is equally dramatic. When the laser is switched off, the contracture tension increases rapidly, rising to the value seen on perfusing the preparation with protected NP.

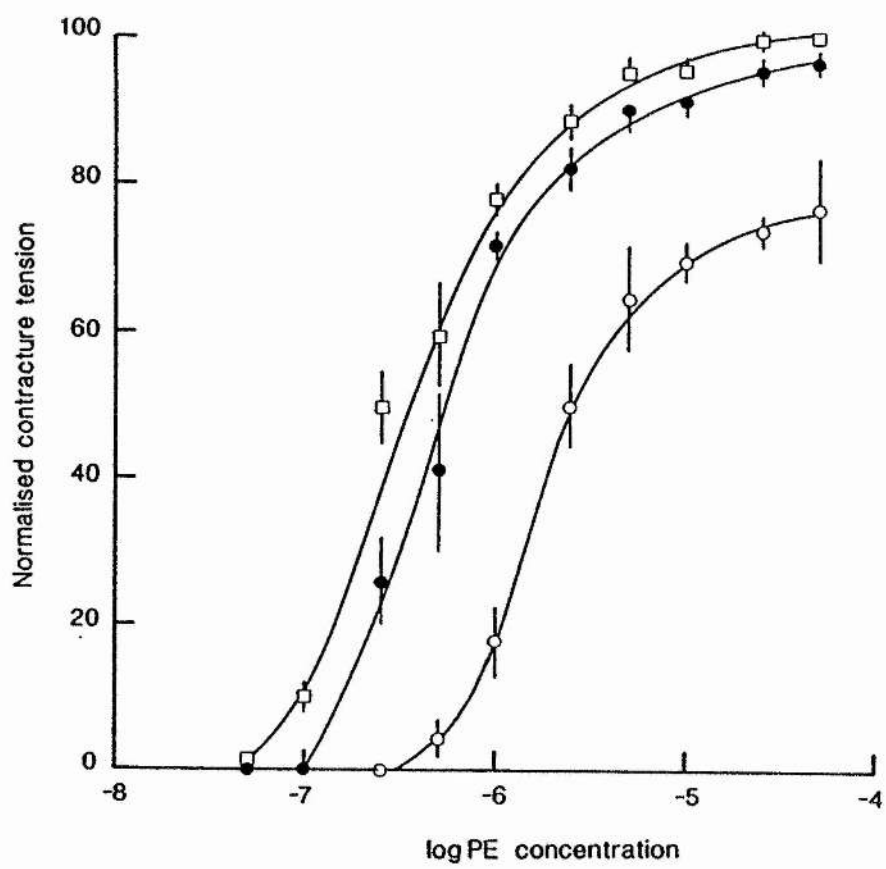
It was found that results obtained using this procedure were identical to those obtained earlier, in the type 1A experiments when NP solutions were exposed to laser (but not white) light. This is illustrated by comparing the third contracture of figure 8:9 with the fifth group of three superimposed contractures shown earlier in figure 8:6. The latter group is reproduced to the right of figure 8:9, after making allowance for the different sizes of the two preparations by scaling the peak contracture tensions in PE alone to the same value. Notice also that the same concentrations of NP (5 $\mu$ M) and of PE (10 $\mu$ M) were used and that the illumination conditions were identical (20mW; 457.9nm). The horizontal dotted lines in figure 8:9 clearly show that the relaxation of pre-contracted strips causes the contracture tension to fall to the same level as that developed by evoking separate contractures in the presence of intact and laser photolysed NP.

The accumulated data from both kinds of experiment are plotted together in figure 8:10. The 'developed' and 'relaxed' contracture tensions are all normalised to the

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### Figure 8:10

The log dose response curve to phenylephrine (white squares), is shown in the presence of  $5 \cdot 10^{-6} \text{M}$  nitroprusside that was either completely protected from, (closed circles), or exposed to, (open circles), 20mW 457nm laser illumination. Each point shows the mean and standard error of 5-8 experiments.





peak values seen with PE alone. The dose response curve to PE (open squares) is slightly shifted to the right with 5 $\mu$ M protected NP (filled circles). The ED<sub>50</sub> value is increased from 0.36 $\mu$ M to 0.55 $\mu$ M, a factor of 1.5 fold. This is qualitatively similar to the displacement seen in a different series of experiments depicted in figure 8:5, although it is somewhat smaller. The most significant features of figure 8:10 are the large rightward shift of the dose response curve and the marked reduction in maximum contracture tensions caused by laser photolysis of NP (open circles). The ED<sub>50</sub> value for PE increases from 0.36 $\mu$ M to 1.7 $\mu$ M, a factor of 4.7 fold, and the maximum contracture tension is reduced to 78% of that obtained with PE alone.

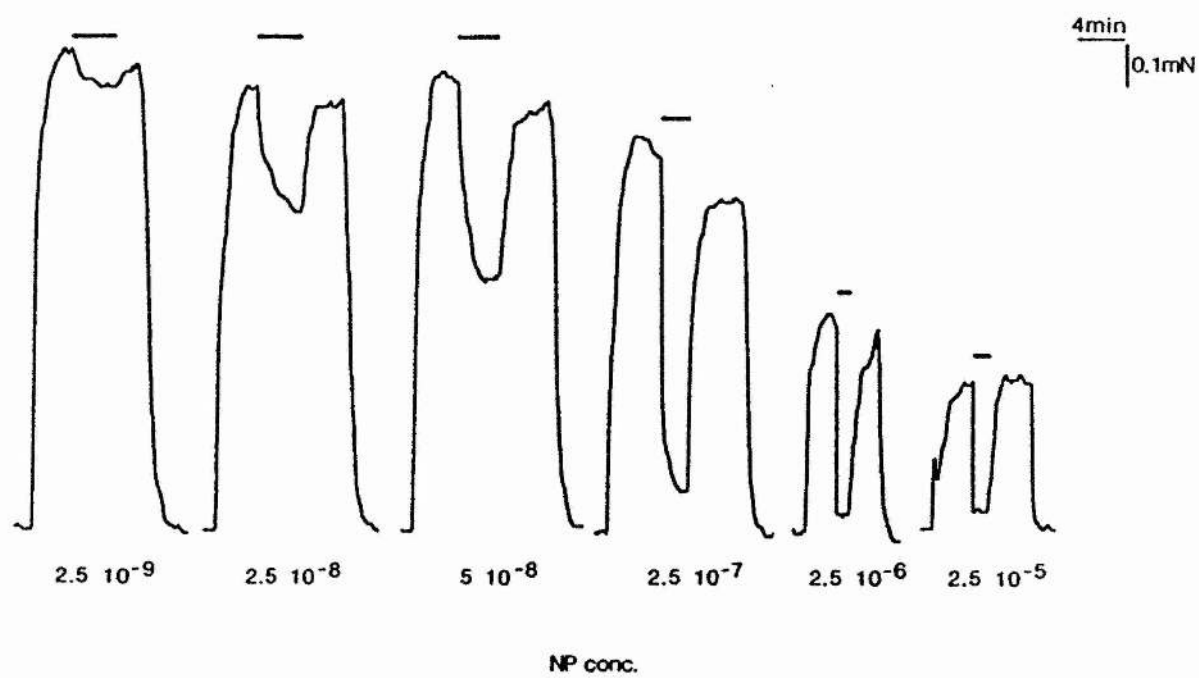
#### Response of arterial strips to combined type 1 & 2 procedures

Figure 8:11 shows representative recordings from an experiment in which contractures to 1 $\mu$ M PE were allowed to develop in the presence of different concentrations of NP, initially protected from light and later, at the peak of the contracture, exposed to laser light (20mW; 457.9nm). Control contractures to 1 $\mu$ M PE alone were performed in between each 'test' contracture to PE + NP. The size of these contractures remained constant and are equivalent to that produced by PE plus 2.5nM protected NP (far left, fig.8:11). The developed tension in the presence of protected NP decreases with increasing NP concentrations, as seen previously (cf. fig.8:7; upper row). The relaxant effect induced by laser photolysis of NP also increases with increasing NP concentrations.

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### Figure 8:11

The effect of laser exposure during a contracture antagonised by intact nitroprusside is illustrated in this diagram. The perfusate was illuminated by 20mW 457nm laser light (depicted by black bar) during contractures elicited by  $10^{-6}\text{M}$  phenylephrine in the presence of various concentrations of unexposed nitroprusside.

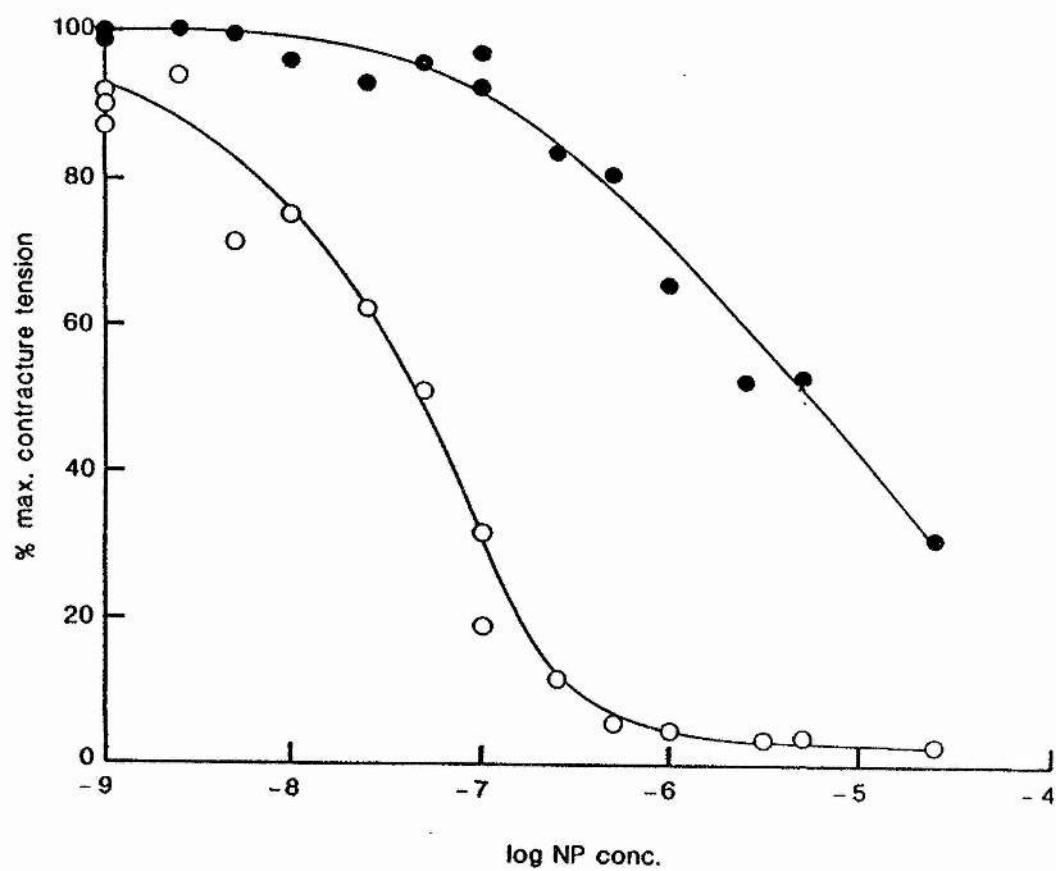


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The results for this and similar experiments are shown in figure 8:12. The filled circles show peak contracture tension in the presence of intact NP, expressed as a percentage of that produced by  $1\mu\text{M}$  PE alone. The open circles show the contracture produced by  $1\mu\text{M}$  PE after the NP is photolysed. The concentration of intact NP producing a 50% reduction in the contracture tension is estimated to be  $5.14\mu\text{M}$ . The comparable figure for the ability of laser photolysed NP to relax the muscle to the same extent is  $45\text{nM}$ . Notice here that  $1\text{nM}$  photolysed NP caused a detectable (10%) relaxation of pre-contracted preparations, whereas the threshold concentration for intact NP is 100 times higher ( $10^{-7}\text{M}$ ).

Figure 8:12

The antagonistic dose response curves for the effects of unexposed (closed circles), or laser photolysed (open circles), nitroprusside on contractures elicited by  $10^{-6}$ M phenylephrine are shown. Each response is normalised to the size of the preceding control contracture to PE alone.



Discussion

The ability of NP to cause relaxation of vascular smooth muscle is the physiological basis for its antihypertensive action in vivo. There is strong evidence to show that this action is linked to its ability to stimulate guanylate cyclase activity to raise intracellular cGMP levels, a property which it shares with the other so-called 'nitrovasodilators' (Rapoport & Murad, 1983; Waldman & Murad, 1987). The nitrovasodilators are aptly named as they are all capable, under appropriate conditions, of forming or releasing nitric oxide which appears to be essential for their activation of guanylate cyclase (Ignarro et al, 1981; see gen. intro.). The enzyme contains a labile haem prosthetic group, and stimulation of its activity by NO is thought to depend on the formation of a nitrosyl-haem.

The experiments presented here show that both protected and photolysed NP can antagonise contracture development to PE and also cause relaxation of pre-contracted arterial strips. The ability of intact NP to effect these changes is especially significant. It is clear that in this respect, vascular smooth muscle is strikingly different to frog ventricular muscle. The latter is completely insensitive to intact NP, even when used at concentrations of  $10^{-2}$ M. In contrast, the threshold effect for protected NP on the development of PE contractures is seen at around 100nm (fig.8:6, 8:12). There are two possible explanations for this difference; vascular smooth muscle may have a mechanism for 'stripping' NO from NP which

is lacking in frog ventricular cells, or alternatively, the responses of vascular smooth muscle are not mediated by NO, but by some other mechanism. The second explanation is not considered likely for two reasons, first, the photolysis of NP, which is known to cause NO release (Wolfe & Swinehart, 1975), greatly enhances its efficacy, both in inhibiting contractures and inducing relaxation. Secondly, evidence in the literature strongly supports the idea that NO mediates the effects of NP ( and other nitrovasodilators) on vascular smooth muscle (see Chapter 1). It therefore seems probable that smooth muscle cells possess reactive groups which are able to interact with NP, extract NO and transfer it to guanylate cyclase. There is evidence that thiols present in the cytosol of smooth muscle are involved in this sequence of events (Ignarro et al, 1981). It is postulated that they react with nitrovasodilators to form an nitrosothiol intermediate which then stimulates GC.

The extreme sensitivity of vascular smooth muscle to NO, implied by the experiments presented here, deserves special mention. Consider the data shown in figure 8:10. Calculations of the amount of NO formed under the conditions used here, using the procedure described in chapter 5, show that this could not have been more than 30nM, yet this is sufficient to cause an almost 5 fold increase in the ED<sub>50</sub> value for PE. Consider also the antagonistic dose response curve of figure 8:12. Similar calculations show that a NO concentration of only 37pM is sufficient to produce a threshold relaxant effect, 805pM induces half relaxation, whereas 5nM completely relaxed an arterial strip



pre-contracted by  $1\mu\text{M}$  PE. These concentrations are lower than the  $0.2\mu\text{M}$  reported in the literature to fully relax a bovine coronary arterial strip precontracted by  $30\text{mM}$  K (Gruetter, Barry, McNamara, Kadowitz & Ignarro, 1981). It is important to emphasise that these values must represent upper estimates, because there is no reliable method of estimating the amount of NO lost through secondary reactions with dissolved oxygen (Wolfe & Swinehart, 1975). The less favourable (ie. highly oxygenated) conditions in this report may explain the discrepancy, as nitric oxide generated by photolysis in these experiments would not be so readily lost.

In connection with this last point, mention should be made of the much greater efficacy of laser photolysed NP as compared to NP pre-exposed to white light. Estimates of the half life of NO in solution range from 6-30 seconds depending on oxygenation and temperature of the solution. Therefore, although the duration of exposure to white light was much greater, and hence the total amount of NP photolysed was greater, it is probable that very little NO survives before being applied to the preparation. Exposure to laser light on the other hand, yields smaller quantities of NO, but it is generated immediately before the solution reaches the muscle and so there is little time for it to react with dissolved oxygen. Spectrophotometric measurements of [Aq] formed during a 1.5hr exposure to white light show that  $0.67\mu\text{M}$  NO is generated from  $5\mu\text{M}$  NP, which greatly exceeds (by 22 times) that produced by laser photolysis of the same solution en route to the chamber ( $30\text{nM}$ ). A

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comparison of the relative efficacies of the two procedures suggest that >95% of the NO formed by pre-exposing NP to white light is consumed prior to use.

## CHAPTER 9

Summary and general discussion.

Summary

(1). A study has been made of the mechanism of action of nitroprusside on isolated frog ventricular trabeculae and rabbit ear arterial strips.

(2). The nitroprusside anion per se has no physiological action on frog ventricle (see chapters 3&4). However, photolysis of nitroprusside results in the formation of a labile photolytic product which has a potent negative inotropic effect. The follow evidence is presented in support of this hypothesis (see chapter 4):-

i. The extent of photolysis of nitroprusside, as determined by the aquapentacyanoferrate concentration, correlates with the degree of depression of the twitch.

ii. Photolysis of nitroprusside has been shown to be dependent on both the intensity and wavelength of the incident light. Higher intensities of illumination, which are more potent in inducing photolysis, produce a greater reduction in twitch tension.

iii. Shorter wavelengths of illumination have a more potent effect than longer wavelengths at the same intensity.

(3). The photolytic product responsible is shown to be labile, as the time between exposure of nitroprusside and application to the muscle is critical in determining the extent of the negative inotropic response (see chapter 3)

(4). The following evidence shows that the physiologically

active photolytic product is nitric oxide (see chapter 6):-

i. Agents which are known to combine with nitric oxide (ie. haemoglobin, superoxide anions or oxygen) are shown to inhibit the response. Nitrogen-flushed solutions augmented the response, whereas oxygen-flushed solutions attenuated it.

ii. The other products of photolysis, namely aquapentacyanoferrate, nitrate and nitrite, have all been shown to be physiologically inactive.

iii. The production of nitric oxide was confirmed by reacting the products of photolysis with haemoglobin. Haemoglobin avidly binds nitric oxide to form nitrosyl-haemoglobin, which was identified by both visible and electron paramagnetic resonance spectroscopy.

(5). The reduction in peak twitch tension is associated primarily with a reduction in the maximum rate of rise of tension, but at more severe depressions, the time to peak tension is also shortened. A reduction in the rate of relaxation is sometimes observed although this is not consistently seen (see chapter 4).

(6). A procedure is described for estimating the concentration of nitric oxide formed from low concentrations of nitroprusside ( $< 10^{-4}M$ ), insufficient to generate detectable quantities of aquapentacyanoferrate. Estimations of the quantal efficiency of photolysis at different wavelengths were obtained during the course of this study (see chapter 5).

(7). Cysteine did not enable nitroprusside to induce a

response without prior exposure to light. However, it potentiated the response to low concentrations of photolysed nitroprusside ( $<10^{-5}$ ), but attenuated the action at higher concentrations (see chapter 7).

(8). The results suggest that cysteine cannot react directly with intact nitroprusside to form nitrosocysteine. It was shown that haemoglobin does not react with intact nitroprusside to produce nitrosyl-haemoglobin (see chapter 6&7).

(9). Synthetic nitrosothiols (nitrosocysteine and s-nitroso-N-acetylpenicillamine) are potent negative inotropic agents (see chapter 7).

(10). The nitroprusside anion per se induces relaxation of precontracted mammalian vascular smooth muscle. However, this effect is markedly potentiated by photolysis (see chapter 8).

(11). It is concluded that the mechanism of action of nitroprusside in both frog ventricle and mammalian arterial smooth muscle involves activation of guanylate cyclase by nitric oxide.

General Discussion

Many of the points raised by this study have already been discussed in the relevant chapters and so will not be repeated here. Instead, the major findings will be discussed in the context of previous reports and in relation to possible future studies.

The major conclusion is that photolysis of nitroprusside (NP) increases its efficacy to decrease tension in both frog ventricle and mammalian vascular smooth muscle. The active photolytic product has been identified as nitric oxide which supports the hypothesis that NP, along with the other 'nitrovasodilators', mediates its action through a nitric oxide intermediate. A wealth of evidence now exists that nitric oxide directly stimulates guanylate cyclase in vascular smooth muscle, which raises cGMP levels and induces relaxation. However, it is difficult to relate the results described here with those previously reported, because the photosensitivity of NP has not been fully appreciated. Over 90% of the reports using NP fail to mention the precautions taken (or otherwise) to protect NP from light. Others (<10%) briefly mention that NP was protected from light, but do not say to what extent. In practice this normally entails preparing the solution in light then wrapping the container in aluminium foil. Unless the experiment is conducted in complete darkness throughout, which is not very practicable, then exposure to light will occur. This has led to the suggestion that nitroprusside spontaneously releases NO in aqueous solution, a phenomenon that can only occur through photolysis.

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It is possible that protection from light is not normally adequate to prevent this.

The extreme photosensitivity of NP is not widely appreciated, as only small intensities of light for brief periods are sufficient to degrade physiologically significant amounts. For example, the brief exposure (<0.5sec) to low intensity (<1.5W) white light described in chapter 3, generates enough NO to elicit a large depression in frog ventricular contractility. As the sensitivity of smooth muscle to NO is very much greater (see chapter 8), then arguably, even less photolysis might be sufficient to influence the physiological response. Previously reported pharmacological and chemical actions of NP may not be due to NP per se, but nitric oxide released through its photochemical destruction.

The lower efficacy of pre-exposed NP as compared to that photolysed in situ, emphasises that the site and timing of illumination is also important. If photolysis occurred before application (eg. during preparation or storage), then a reduction in efficacy may ensue, as the concentration of both intact NP and liberated NO will decrease. This will depend on the time interval between illumination and application, as well as on the level of oxygenation of the solution. On the other hand, photolysis in situ (eg. in the organ bath or enzyme assay) or just before application, might potentiate the response, as the released NO would interact directly at the site of action. The usual assumption that photolysis leads to a decrease in efficacy is therefore not true.

In view of the above, the results of previous reports using NP must be interpreted with caution. Very rarely are



adequate precautions taken to prevent photolysis, and this has led to difficulty in interpreting results. In future studies, experiments should be made with both protected and photolysed NP.

Inadvertent photolysis of NP may have led to incorrect conclusions about its pharmacological reactions. A recent example is in a study comparing the antagonistic effects of cyanide and methylene blue on arterial relaxation induced by EDRF and some nitrovasodilators (NP, GTN & S-nitroso-N-acetylpenicillamine) (Ignarro, Harbison, Wood & Kadowitz, 1985). It was found that methylene blue antagonised relaxation by all these relaxants, but cyanide only inhibited responses to NP. It was proposed that cyanide was 'chemically inactivating' NP as preincubation of cyanide with NP inhibited its ability to relax smooth muscle. However, this only occurs if cyanide and NP are incubated at 37°C for 12-15 minutes in a highly oxygenated Krebs solution. A similar incubation in low oxygenated solution was ineffective. Photolysis of NP could explain these results. There was no mention of any precautions to protect NP from light, and it was claimed that NP "releases nitric oxide gas". The decrease in efficacy may be due to a decrease in NP concentration through degradation. In high oxygen the NO released would be rapidly scavenged, whereas more NO would remain in solution in low oxygen, which could explain why the effect was not seen in low oxygen conditions. Also, the reported change in the spectral properties of preincubated NP would be consistent with the production of aquapentacyanoferrate. It appears therefore that cyanide must antagonise the relaxant action of NP by some other mechanism

(possibly by binding to intracellular haem) than by chemically inactivating NP. This is substantiated by Leeuwenkamp et al (1986), who showed that cyanide did not affect NP breakdown by crude aortic soluble fraction in the dark.

Although this study has been highlighted, it is by no means unique. In general, the extent of photolysis and its influence on experimental results cannot be assessed.

An important conclusion to be drawn from the present study is that frog ventricle is insensitive to intact NP, but mammalian smooth muscle is not. Evidence in the literature suggests that thiol or haem groups in smooth muscle are responsible for cleaving NO from NP (see gen.intro.). Most of the evidence for this is plagued with doubts concerning the influence of photolysis. For example, the reported 'spontaneous' formation of nitrosocysteine or nitrosohaemoglobin when NP is added to either cysteine or haemoglobin (Hb), has been used as evidence for this hypothesis. However, results presented here (chapters 6&7) show that Hb can form nitrosyl-haemoglobin only with photolysed NP. Indirect evidence is also presented that intact NP and cysteine do not interact, as this mixture fails to have any effect on a preparation shown to be sensitive to preprepared nitrosocysteine (chapter 7). These results suggest that prior photolysis may be responsible for the in vitro formation of these nitrosocompounds. This does not of course, preclude their possible formation in vivo, as there may be a tissue co-factor/s (ie. a reducing agent) which mediates nitrosyl cleavage from NP. Direct evidence for this is the reported cleavage of NP by crude aortic soluble fraction in

the dark (Leeuwenkamp et al, 1986).

Since frog ventricle is unresponsive to intact NP, it is postulated that this tissue lacks the ability to degrade NP. Photolysis however, releases nitric oxide which then induces a potent negative inotropic effect. It was previously shown that this correlates with an elevation of cGMP levels in hypodynamic frog heart. This led to the suggestion that cGMP is involved directly in regulating the inotropic status of the frog heart. If this hypothesis is correct, then it follows that intact NP should not alter intracellular cGMP. However, preliminary experiments carried out during the course of this study showed that both intact and pre-exposed NP (see chapter 3) raised [cGMP] 2-4 fold, although only photolysed NP depressed the twitch. This is the first indication to date that cGMP may not be as directly involved in regulating contractility as previously thought. These experiments were conducted on normal (non-hypodynamic) ventricles using pre-exposed NP. For reasons given earlier (see chapter 3), these conditions are not ideal and further studies are therefore required. Levels of cGMP in normal and hypodynamic ventricles in response to treatment with intact or photolysed (in situ) NP should be measured before any definitive conclusions can be drawn. It may be that frog ventricle has physiologically distinct pools of cGMP which mediate different functions, as has been suggested for mammalian heart. Interestingly, preliminary experiments have shown that atrial natriuretic peptide (ANP), an agent known to stimulate particulate guanylate cyclase, has no effect on frog ventricle. Thus, either the ANP receptor or particulate GC is

absent in frog, or else if present then it is coupled to an intracellular pool of cGMP which is not associated with the regulation of contraction. Measurements of [cGMP] in the presence of ANP are required before any conclusions can be drawn.

The effects of photolysis were also briefly investigated in mammalian cardiac preparations. Neither photolysed nor intact NP had any effect on the contractility of mouse papillary muscle or rat ventricular trabeculae, which is in agreement with previous studies. Preliminary measurements on isolated rabbit ventricular myocytes, showed that both intact and photolysed NP elevated cGMP levels 11-13 fold (in the presence of IBMX). It would appear, then, that photolysis is not necessary to stimulate GC activity in mammalian heart. The function of cGMP in this tissue remains enigmatic and further studies, possibly using specific phosphodiesterase inhibitors, are required in order to recognise these discrete cGMP pools and identify their physiological function.

The recent identification of EDRF as nitric oxide and the discovery of ANP has led to renewed interest in the role of guanylate cyclase and cGMP in regulating both cardiac and vascular smooth muscle contractility. The discovery of an endocardial-derived contractile factor in mammalian heart is also of considerable interest. It is likely that these developments will prove to be of great significance in understanding the pathogenesis of various cardiovascular disorders, especially hypertension, and that they will result in new strategies in the design of improved methods of treatment.

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# **Trifluoperazine suppresses the 'phasic' component of the K<sup>+</sup> contracture in frog atrial trabeculae**

By F. W. FLITNEY and G. KENNOVIN. *Department of Physiology and Pharmacology, University of St Andrews, St Andrews, Fife KY16 9TS*

Trifluoperazine (TFP; Smith, Kline & French Ltd, Welwyn Garden City, Herts) is an inhibitor of Ca<sup>2+</sup>-activated calmodulin (Levin & Weiss, 1977). An earlier report (Flitney, Moshiri, Robertson & Singh, 1981) showed that TFP depresses twitch contractions of frog ventricular muscle. In this communication we demonstrate that it also depresses K<sup>+</sup> contractures of frog atrial trabeculae, and that it inhibits the phasic component preferentially. Trabeculae were mounted in a perfusion chamber

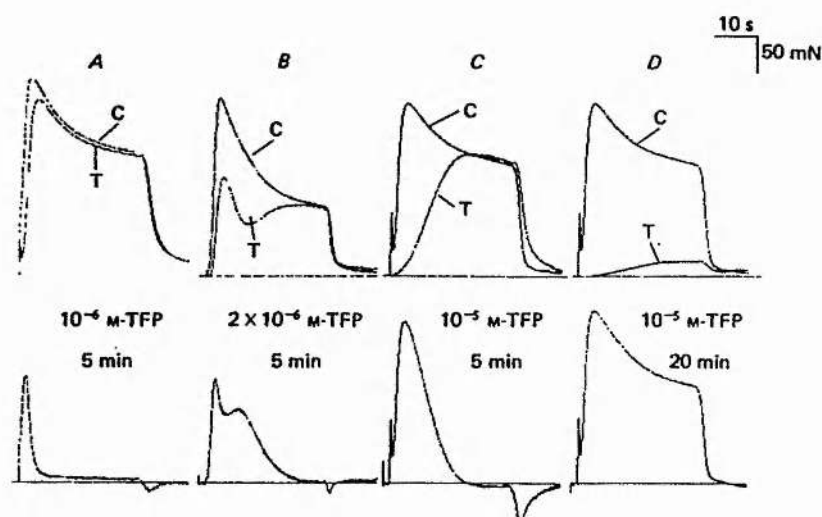


Fig. 1. A-D, pairs of contractures (test and controls; upper records), together with traces highlighting the differences between the two (control minus test; lower records). The effect of TFP increases with time (C, D) and is fully reversible. It is dose-dependent (A-C) and preferentially depresses the phasic component (C). The latter effect is half-maximal at 1-2  $\mu$ M (measured after 5 min exposure), under which conditions the effect on the tonic component is negligible.

and attached to a strain gauge using tungsten-wire snares. They were stimulated electrically (0.2 Hz; 3-12 V; 1-5 ms) and the rest length adjusted to give a maximal twitch. Contractures were elicited (30 s duration, 15 min intervals) using Ringer's solution containing 200 mM-KCl. The effects of TFP were tested by pre-treating preparations with concentrations ranging from 10<sup>-7</sup> to 10<sup>-5</sup> M for 5-65 min.

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# Photochemical degradation of nitroprusside in relation to its negative inotropic action on frog heart

By F. W. FLITNEY and G. KENNOVIN. *Department of Physiology and Pharmacology (Westburn Annexe), University of St Andrews, Fife KY16 9TS*

The nitroprusside anion ( $[\text{Fe}(\text{CN})_5\text{NO}]^{2-}$ ) is stable in the dark but readily undergoes photolysis when exposed to light (Wolf & Swinehart, 1975). The experiments in Fig. 1 show that its negative inotropic effect on frog heart (Flitney, Moshiri & Singh, 1980*a, b*) is probably caused by a photodegradation product. The experiments were conducted in a darkened room with a red safe-light as the only means of illumination. Isolated ventricular trabeculae were used from specimens of *Rana temporaria*.

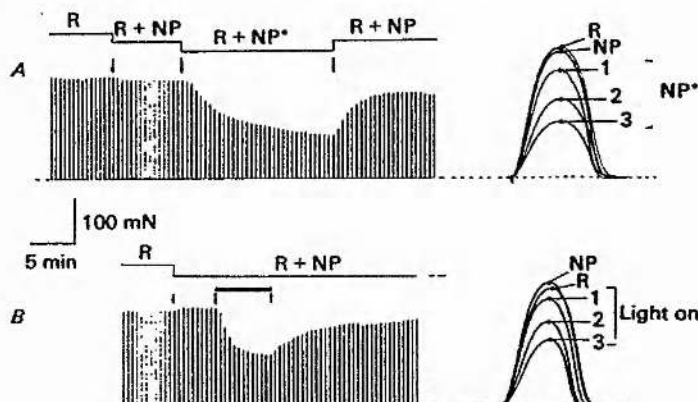


Fig. 1. *A*, a preparation was exposed to  $10^{-2}$  M-NP, freshly prepared and protected from light, with little effect on the twitch. The perfusion fluid was then changed for one containing NP which had been pre-exposed to light (60 W tungsten bulb at 20 cm for 16 h) to ensure its photolysis (denoted NP\*). Peak twitch tension declined (to 42 % of control) and recovered on re-perfusing with  $10^{-2}$  M protected NP. *B*, a preparation was first perfused with protected NP, which again had little effect on the twitch. Light generated by a tungsten halogen source (150 W) was then directed onto the preparation for 5 min using a fibre-optics guide. Twitch tension declined during the period of illumination.

The experiments show that exposure of NP to light is essential to elicit a negative inotropic response. NP is known to be a potent activator of the enzyme guanylate cyclase (Murad, Arnold, Mittall & Braughler, 1979) and our previous experiments showed that it increased cyclic GMP levels in frog heart. The present observations therefore raise an interesting question: is the stimulation of guanylate cyclase also dependent upon a photodegradation product? If so, photoactivation of guanylate cyclase (by illuminating preparations previously loaded with protected NP) could permit intracellular cyclic GMP levels to be increased abruptly.

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# **Laser-induced photolysis of nitroprusside: response of isolated frog ventricular trabeculae to photodegradation products**

By F. W. FLITNEY and G. KENNOVIN. *Department of Physiology, University of St Andrews, St Andrews, Fife KY16 9TS*

Twitch contractions of frog ventricular trabeculae are unaffected by nitroprusside (NP;  $[\text{Fe}(\text{CN})_5\text{NO}]^{2-}$ ) but are depressed by products which are generated during its photolysis (Flitney & Kennovin, 1985). Here we demonstrate that the response is quantitatively related to the spectral efficacy of the photolytic process. Preparations were superfused with NP and with NP which had been illuminated (*en route* to the muscle chamber) with monochromatic light from an Argon ion laser ( $\lambda$  (nm) = 514.5, 488, 457.9).

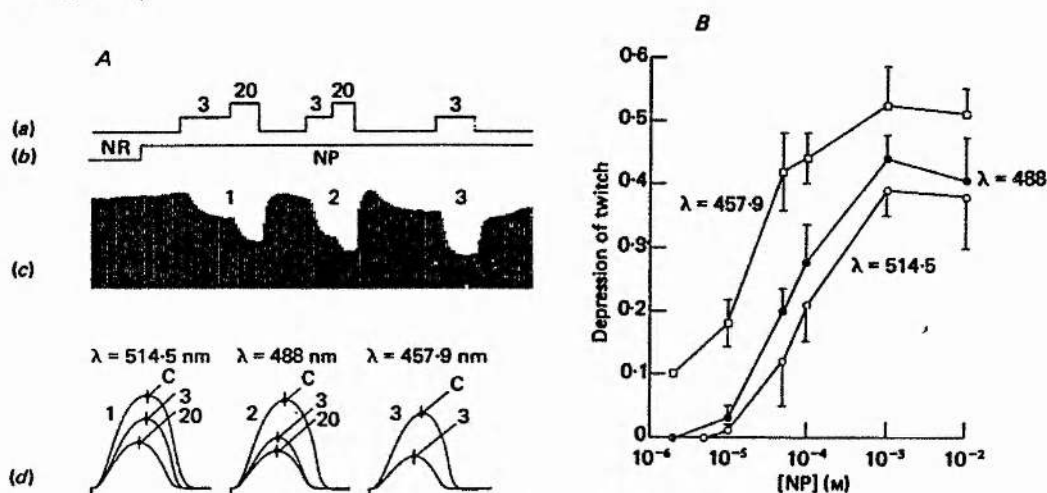


Fig. 1. *A*, Effects of varying intensity of illumination and  $\lambda$  on responses to photolysed NP: (a) pattern of illumination (numbers = light intensity (mW)); (b) solution changes; NR = normal Ringer; (c) twitch tension; (d) sample twitches (C = control; 3, 20 = mW). *B*, Log dose-response curves for NP exposed to 3 mW laser light at  $\lambda$ s indicated.

The results (Fig. 1*A*, *B*) show that the effect on the twitch (*a*) increases with increasing light intensity and (*b*) varies inversely with  $\lambda$ . The  $\text{ED}_{50}$  values estimated from Fig. 1*B* (and from similar dose-response curves, not shown) are reciprocally related to both the intensity of illumination (*I*) and the quantal efficiency ( $\Phi$ ) of photolysis.

These observations reinforce our earlier conclusion that responses of the frog ventricle previously attributed to 'nitroprusside' (e.g. Flitney, Moshiri & Singh, 1980) are in reality caused by one (or more) of its photodegradation products.

We thank the Wellcome Trust and British Heart Foundation for support, and Mr W. Burke (Burke Electronics Ltd, Glasgow) for lending us the laser used in this study.

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**Is the stimulation of guanylate cyclase by nitrogen(II) oxide responsible for the negative inotropic effect of 'nitroprusside' on frog ventricular trabeculae?**

By F. W. FLITNEY and G. KENNOVIN. *Department of Physiology, University of St Andrews, St Andrews, KY16 9TS*

Twitch contractions of frog ventricular trabeculae are unaffected by the nitroprusside anion (pentacyanonitrosylferrate(II) or  $[\text{Fe}^{\text{II}}(\text{CN})_5\text{NO}]^{2-}$ ) but are depressed by products formed during its photolysis (Flitney & Kennovin, 1986). The absorption of a photon (by  $\text{Fe}^{\text{II}}$  ligand) results in pentacyanoferrate(III) and nitrogen(II) oxide ('NO') formation (Wolf & Swinehart, 1975). The subsequent fate of 'NO' is oxygen-dependent whereas that of pentacyanoferrate(III) is not. The ['NO'] can therefore be altered by varying  $[\text{O}_2]$  in the perfusate. Log dose-response curves to laser-photolysed nitroprusside (NP) are shown in Fig. 1 for nitrogen-, air- and oxygen-flushed solutions.

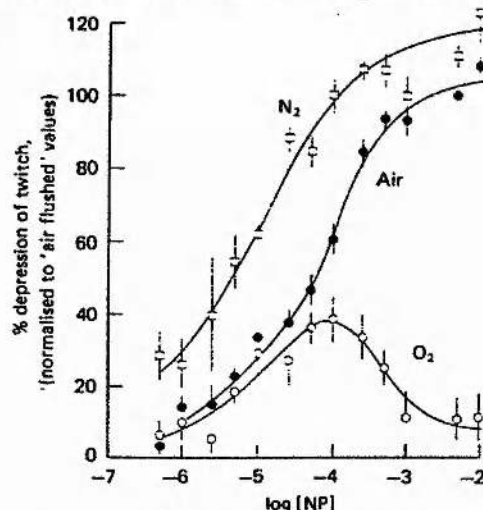


Fig. 1. Dose-response curves to NP in solutions flushed with  $\text{N}_2$ , air and  $\text{O}_2$ . NP solutions exposed to laser light ( $\lambda = 457.9 \text{ nm}$ ,  $20 \text{ mW}$ ) *en route* to the chamber. Flow rate =  $9.5 \text{ ml min}^{-1}$ . Means  $\pm$  s.e.s,  $n = 6-8$  preparations.

The  $\text{N}_2$  curve ( $[\text{O}_2] = 0.11 \text{ mmol l}^{-1}$ ) is displaced to the left relative to the air curve ( $[\text{O}_2] = 0.35 \text{ mmol l}^{-1}$ ). The high  $\text{O}_2$  curve ( $[\text{O}_2] = 1.25 \text{ mmol l}^{-1}$ ) is similar to that for air at  $[\text{NP}]$ 's  $< 100 \mu\text{M}$  but responses are markedly depressed at higher  $[\text{NP}]$ 's. These results provide evidence that 'NO' is the active species and that it exerts its effects via the enzyme guanylate cyclase (GC). This interpretation is consistent with the known effects of 'NO' on purified GC, which displays a bell-shaped dose-activity relationship (Murad, Arnold, Mittal & Braugher, 1979).

We thank the Wellcome Trust and British Heart Foundation for supporting this study.

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# **Photolysis of nitroprusside enhances its relaxant effect on rabbit arterial smooth muscle**

By F. W. FLITNEY and G. KENNOVIN. *Department of Physiology, University of St Andrews, St Andrews KY16 9TS*

Nitroprusside (NP) must first be photolysed before it can exert a depressant effect on twitch contractions of frog ventricular trabeculae (Flitney & Kennovin, 1986). The experiments to be described will show that the situation is different for mammalian smooth muscle. Strips of rabbit ear artery were made to contract with phenylephrine (PE) and then perfused with solutions which also contained NP. The latter were kept in the dark initially but then exposed for 1–2 min to laser light ( $\lambda = 457.9$  nm, 20 mW) *en route* to the muscle chamber.

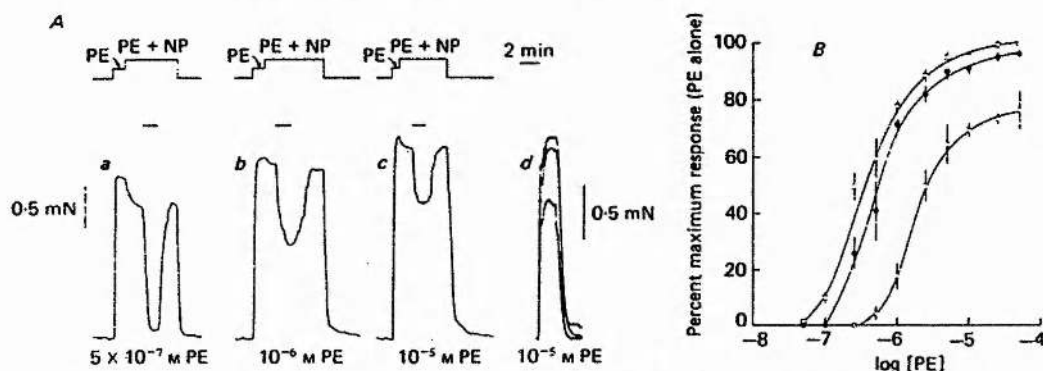


Fig. 1. *A*. Individual responses to PE and to protected or laser-photolysed (horizontal bar) NP. *B*. Log dose-response curves for PE alone (□), PE + protected NP (●) and PE + exposed NP (○). Traces in *d*, consecutive contractions (superimposed) showing similar responses to those induced during single contractions (*a*–*c*). Means  $\pm$  S.E.,  $n = 5$  preparations.

Fig. 1 *A* shows that *protected* NP causes a small relaxation. This effect is greatly enhanced during exposure to light (horizontal bars). Dose-response curves to PE in the presence of  $5 \mu\text{M}$ -NP (Fig. 1 *B*) show that the efficacy of NP is increased ca. 3–5 × following its photolysis.

We thank the Wellcome Trust and British Heart Foundation for supporting this work.

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**Haemoglobin suppresses the negative inotropic effect of photolysed nitroprusside on isolated frog ventricular trabeculae: identification of nitrosyl-haem formation by electron paramagnetic resonance and absorption spectroscopy**

BY F. W. FLITNEY and G. KENNOVIN. *Department of Biology and Preclinical Medicine, University of St Andrews, St Andrews KY 16 9TS, Fife, Scotland*

Photolysis of nitroprusside (NP) generates a reaction product which depresses twitch contractions of frog ventricular trabeculae. Evidence was presented earlier (Flitney & Kennovin, 1987) implicating nitric oxide ( $\text{NO}^*$ ) as the physiologically active species formed. The experiments now reported show that the inotropic effect can be attenuated and may be suppressed entirely by haemoglobin (Hb) in  $\mu\text{M}$  concentrations (Fig. 1A). The haem prosthetic group reacts with  $\text{NO}^*$  under the conditions of our experiments to form nitrosyl-haem. This has been identified (a) by splitting of the 555 nm absorption band of deoxyhaemoglobin (Fig. 1B) and (b) by the appearance of an electron paramagnetic resonance (EPR) signal (Fig. 1C) showing the hyperfine structure characteristic of highly protonated nitrosyl-haem (Trittelvitz *et al.* 1977).

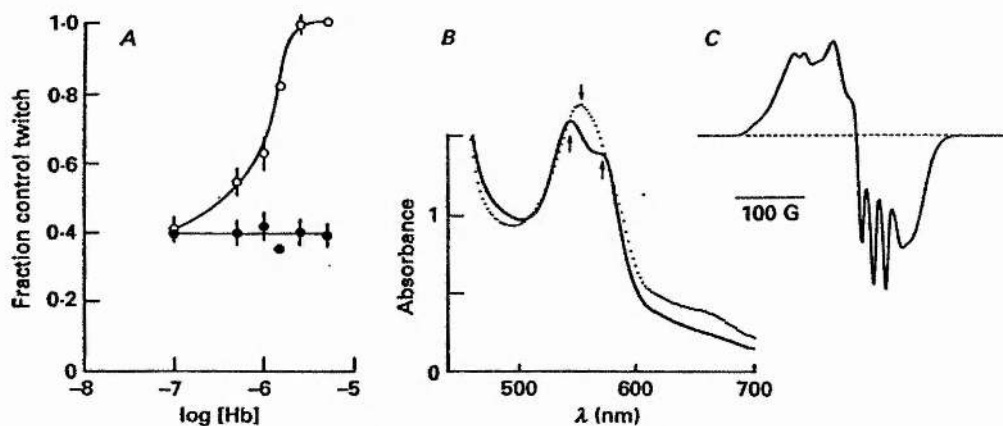


Fig. 1. A, responses to 1 mM-NP exposed to laser light ( $\lambda = 457.9$  nm, 20 mW) without ( $\bullet$ ) and with Hb ( $\circ$ ). B, splitting of 555 nm absorption maximum of deoxy-Hb on binding of  $\text{NO}^*$  ligand. C, EPR spectrum (17 K, scan 1000 G, mid-scan 3500 G; in 100 mM-phosphate buffer, pH 7 at 20 °C) showing hyperfine structure typical of nitrosyl-haem.

These results support our earlier contention that  $\text{NO}^*$  is the cardioactive species formed during photolysis of NP.

We thank the Wellcome Trust and British Heart Foundation for grant support and Dr J. Ingledew and Mr R. Rotherby for EPR facilities.

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